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(54) Title: HCV NS2/3 FRAGMENTS AND USES THEREOF

(57) Abstract: Truncated HCV NS2/3 can be produced recombinantly and renatured into functional enzyme, useful in assays for modulators of activity and HCV function.

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HCV NS2/3 FRAGMENTS AND USES THEREOF

The present invention relates to assays, screening methods, polypeptides, mimetics, and methods of use based on a
5 polypeptide derived from Hepatitis C virus (HCV) NS2/3 protease.

The hepatitis C virus (HCV) is the major causative agent of parenterally-transmitted and sporadic non-A, non-B hepatitis
10 (NANB-H). Some 1% of the human population of the planet is believed to be affected. Infection by the virus can result in chronic hepatitis and cirrhosis of the liver, and may lead to hepatocellular carcinoma. Currently no vaccine nor established therapy exists, although partial success has been
15 achieved in a minority of cases by treatment with recombinant interferon-α, either alone or in combination with ribavirin. There is therefore a pressing need for new and broadly-effective therapeutics.

20 Several virally-encoded enzymes are putative targets for therapeutic intervention, including an autoprotease (NS2-3), a serine protease (NS3), a helicase (NS3), and an RNA-dependent RNA polymerase (NS5B).

25 The NS3 protease domain is located at the N-terminal of the NS3 protein, and is responsible for an intramolecular cleavage at the NS3/4A site and for downstream intermolecular processing at the NS4A/4B, NS4B/5A and NS5A/5B junctions.

30 NS3 is formed by the proteolytic cleavage of the precursor molecule NS2/3, generating the mature N-terminal of NS3. This reaction is auto-catalysed by the NS2/3 precursor molecule itself.

The auto-catalytic activity of the NS2/3 protease is believed to be essential for the replication of HCV (Kolykhalov, A. *et al.* (2000) J. Virol. 74 2046-2051)and therefore represents a potentially important therapeutic target in the treatment of

5 HCV infection.

The precise mechanism of NS2/3 cleavage is unknown but it is known to be an intra-molecular, possibly co-translational, reaction that is catalyzed by the NS2/3 proteolytic activity

10 (Wu *et al.*, TIBS 23, 92-94, 1998). Because the activity is stimulated by the addition of metal ions such as Zn or Cd to *in vitro* translation assays, the enzyme has been tentatively classified as a metalloprotease. Study of the NS2/3 precursor has been complicated by its auto-proteolytic activity,

15 insolubility and instability.

No *in vitro* assays using a purified NS2/3 protease have yet been reported. The insolubility and instability of the protease and its auto-catalytic activity have blocked the

20 development of such assays.

NS2/3 protease activity in cell-free translation systems and in transfected cells has been described by Grakoui *et al* (1993)PNAS 90, 10583-10587, and Hijikata *et al.*(1993) J. Virol

25 67, 4665-4675. The cleavage site of the NS2/3 protease was found by these groups to be between residues 1026 and 1027 of the HCV polypeptide and cleavage was shown to be Zn-dependent. Cys993 and His952 were determined to be essential for cleavage and sequences between residues 827 and 1186-1207 were also

30 required for activity. A trans-cleavage activity was also identified. All the studies conducted by these groups were on cells or cell extracts.

Reed *et al.* (1995) J. Virol. 69, 4127-4136 reported the trans-
cleavage activity and trans-inhibition of cleavage of the
NS2/3 protease in cell-based systems and also described the
sequence specificity of the cleavage reaction.

Pieroni *et al* (1997) J. Virol 71, 6373-6380 described the
production of NS2/NS3 precursor in a latent form in a cell-
free translation system and also its reactivation by the
addition of detergent. However, the system described did not
involve purified enzyme.

Cleavage at the NS2/3 site requires both the NS3 protease
domain (but not its serine protease activity) and the NS2
protein that starts at residue 810 of the HCV NS2/3 precursor.
The first ~ 100 residues of NS2 are highly hydrophobic and may
be associated with the ER membrane in infected cells. This
hydrophobic portion may be responsible for the poor solubility
of the protein. Deletions of the N-terminal region up to
residue 923 have been shown to abolish activity.

Based on the experimental work and discussion herein, the
present invention in various aspects is based on the obtaining
NS2/3 protease polypeptides that start at a residue from 903
to 913 i.e. 903, 904, 905, 906, 907, 908, 909, 910, 911, 912,
or 913, and terminate at residue 1206, residue 1657 or a
residue between 1206 and 1657.

Surprisingly, these truncated polypeptides retain the auto-
proteolytic activity of the full length protease but are
capable of undergoing purification in the precursor form
without undergoing auto-cleavage. Furthermore, it is a
dimeric form of the polypeptide which is shown to be

responsible for the auto-proteolytic activity. The active
polypeptide is therefore capable of dimerisation to generate
the auto-proteolytic activity.

5   The provision of an active precursor molecule in turn enables
the provision of assays and screening methods for agents that
can modulate, especially inhibit, the auto-proteolytic
activity of the NS2/3 precursor and which therefore have
therapeutic potential in the treatment of HCV.

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Various aspects of the present invention provide for a
polypeptide which has auto-proteolytic activity and which is
capable of being expressed in an inactive form without self-
cleavage and subsequently activated. The auto-proteolytic

15 form of the polypeptide may be a homo-dimer.

The present invention provides a polypeptide or a polypeptide
fragment having an N-terminal boundary between residues 903
and 913 and a C-terminal boundary at or between residues 1206
and 1657. The polypeptide or polypeptide fragment may

20 therefore be truncated at its C terminal or it may comprise
the complete NS3 sequence. Preferably, the polypeptide or
polypeptide fragment includes the complete protease domain of
NS3. In preferred embodiments, the C-terminal boundary is at

25 residue 1206.

References herein to the HCV polyprotein sequence and residues
therein refer to one or more of the HCV polyprotein sequences
from the databases as follows: HCV J strain polyprotein,

30 Swissprot Acc No:P26662; HCV H strain polyprotein, Swissprot
Acc No: P27958; H77 strain polyprotein, Translated-Genbank
Acc. No AAB66324 or TREMBL Acc. No.O36579. However, isolates

of other HCV strains and genotypes may also be employed in
accordance with the present invention.

HCV isolates may be derived from HCV of 1a, 1b, 1c, 2a, 2b,
5 2c, 2d, 2e, 2f, 3a, 3b, 3c, 3d, 3e, 3f, 4a, 4b, 4c, 4d, 5a,
6a, 6b, 7a, 7b, 8a, 8b, 9a, 9b, 9c, 10a or 11a genotype, as
described in Tokita, M. et al J. Gen. Virol.(1996) 77, 293-301
and Myakawa, Y. et al Molecular Med. Today (1995) 1, 20-25, or
derived from HCV of the H-FDA, H-AP, HCV-1, HCV-J, HCV-BK, HC-
10 J6, HCV-T, HC-J8 or HCV-JT strains described in Grakoui et al
Proc. Natl. Acad. Sci. USA (1993) 90, 10583-10587.

The present invention also provides a polypeptide or a
polypeptide fragment consisting essentially of an amino acid
15 sequence starting at residue 903, 913 or a residue located
between these residues and ending at residue 1206, 1657 or a
residue located between these residues. The polypeptide or
polypeptide fragment may therefore be truncated at its C
terminal or it may comprise the complete NS3 sequence.
20 Preferably, the polypeptide or polypeptide fragment includes
the complete protease domain of NS3.

The polypeptide or polypeptide fragment may, for example,
have an N-terminal boundary at residue 903, 904, 905, 906,
25 907, 908, 909, 910, 911, 912 or 913 of the HCV polyprotein and
a C terminal at or between residues 1206 and 1657, for
example, residue 1250, 1300, 1350, 1400, 1450, 1500,1550,
1600 or 1650.

30 A polypeptide may be in isolated and/or purified form, free or
substantially free of material with which it may be
associated, such as other polypeptides, or (for example if
produced by expression in a prokaryotic cell) lacking in

native glycosylation, e.g. unglycosylated.

Polypeptides which are amino acid sequence variants,
derivatives or mutants are also provided by the present
5   invention. A polypeptide which is a variant, derivative or
mutant may have an amino acid sequence which differs from the
corresponding sequence of the HCV polyprotein (for example,
Swissprot Acc No:P26662 described above) by one or more of
addition, substitution, deletion and insertion of one or more
10   amino acids but has auto-proteolytic activity in a dimeric
form and can be expressed in an inactive form and subsequently
activated.

Amino acid sequences may consist of between 293 and 754
15   residues and correspond to the sequence starting at or between
residues 903 and 913 and extending to a sequence at or between
residues 1206 and 1657 of the native HCV protease. More
preferably, sequences may consist of between 293 and 303
residues, corresponding to the sequence starting at or between
20   residues 903 and 913 and extending to residue 1206 of the
native HCV protease.

A polypeptide which is an amino acid sequence variant,
derivative or mutant of the corresponding region of the HCV
25   polyprotein sequence may comprise an amino acid sequence which
shares greater than about 60% similarity, greater than about
70% similarity, greater than about 80% similarity, greater
than about 90% similarity, greater than about 95% similarity
or substantially identical with the amino acid sequence of HCV
30   NS2/3 polyprotein. Amino acid similarity is generally defined
with reference to the algorithm GAP (Genetics Computer Group,
Madison, WI) as noted above, or the TBLASTN program, of
Altschul et al. (1990) J. Mol. Biol. 215: 403-10. Similarity

allows for "conservative variation", i.e. substitution of one
hydrophobic residue such as isoleucine, valine, leucine or
methionine for another, or the substitution of one polar
residue for another, such as arginine for lysine, glutamic for
5   aspartic acid, or glutamine for asparagine. Particular amino
acid sequence variants may differ from a sequence referred to
herein by insertion, addition, substitution or deletion of 1
amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-
150, or more than 150 amino acids.

10

Sequence comparison may be made over the full-length of the
relevant sequences shown herein.

As is well-understood, homology at the amino acid level is
15   generally in terms of amino acid similarity or identity.
Similarity allows for "conservative variation", i.e.
substitution of one hydrophobic residue such as isoleucine,
valine, leucine or methionine for another, or the substitution
of one polar residue for another, such as arginine for lysine,
20   glutamic for aspartic acid, or glutamine for asparagine.
Homology may be taken over the full-length of a sequence or
over a part, such as 20, 30, 40, 50, 60, 70, 80, 90, 100, 120,
150, 200 contiguous nucleotides or amino acids. That two
nucleotide sequences are said to share "homology" or be
25   "homologous" is based on sequence comparison. Any
phylogenetic relationship is irrelevant for this. Those
skilled in the art routinely refer to homology between
nucleotide sequences with no implication for evolutionary
origin. Two homologous nucleotide sequences may also be said
30   to be "similar" or have a certain percentage similarity or a
certain percentage identity.

In general it is not critical which of the various standard

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algorithms are used to determine how homologous two nucleotide
sequences are with one another. A preferred algorithm may be
GAP, which uses the alignment method of Needleman and Wunsch
(*J. Mol. Biol.* (1970) *48*, 443-453) and is included in the
5   Program Manual or the Wisconsin Package, Version 8, September
1994, Genetics Computer Group, 575 Science Drive, Madison,
Wisconsin, USA). In the absence of instructions to the
contrary, the skilled person would understand to use the
default parameters with the aim of maximizing alignment, with
10   a gap creation penalty = 12 and gap extension penalty = 4.

Similarity or homology (the terms are used interchangeably) or
identity may be as defined and determined by the TBLASTN
program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10,
15   or BestFit, which is part of the Wisconsin Package, Version
8, September 1994, (Genetics Computer Group, 575 Science
Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably
sequence comparisons are made using FASTA and FASTP (see
Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98).
20   Parameters are preferably set, using the default matrix, as
follows: Gapopen (penalty for the first residue in a gap): -
12 for proteins / -16 for DNA; Gapext (penalty for additional
residues in a gap): -2 for proteins / -4 for DNA; KTUP word
length: 2 for proteins / 6 for DNA.
25

Nucleic acid sequence homology may be determined by means of
selective hybridisation between molecules under stringent
conditions.

30   Preliminary experiments may be performed by hybridising under
low stringency conditions. For probing, preferred conditions
are those which are stringent enough for there to be a simple
pattern with a small number of hybridisations identified as

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positive which can be investigated further.

For example, hybridizations may be performed, according to the
method of Sambrook et al. (below) using a hybridization
5   solution comprising: 5X SSC (wherein >SSC= = 0.15 M sodium
chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt=s reagent,
0.5-1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm
DNA, 0.05% sodium pyrophosphate and up to 50% formamide.
Hybridization is carried out at 37-42□C for at least six
10   hours. Following hybridization, filters are washed as
follows: (1) 5 minutes at room temperature in 2X SSC and 1%
SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1%
SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4)
2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution
15   every 30 minutes.

One common formula for calculating the stringency conditions
required to achieve hybridization between nucleic acid
molecules of a specified sequence homology is (Sambrook et
20   al., 1989): $T_m = 81.5°C + 16.6Log [Na+] + 0.41 (\% G+C) - 0.63$
(% formamide) $- 600/\#bp$ in duplex.

As an illustration of the above formula, using [Na+] = [0.368]
and 50-% formamide, with GC content of 42% and an average
25   probe size of 200 bases, the T_m is 57°C. The T_m of a DNA
duplex decreases by 1 - 1.5°C with every 1% decrease in
homology. Thus, targets with greater than about 75% sequence
identity would be observed using a hybridization temperature
of 42°C. Such a sequence would be considered substantially
30   homologous to the nucleic acid sequence of the present
invention.

It is well known in the art to increase stringency of

hybridization gradually until only a few positive clones
remain. Other suitable conditions include, e.g. for detection
of sequences that are about 80-90% identical, hybridization
overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10%
5   dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1%
SDS. For detection of sequences that are greater than about
90% identical, suitable conditions include hybridization
overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10%
dextran sulfate and a final wash at 60□C in 0.1X SSC, 0.1%
10   SDS.

The skilled person can use the techniques described herein and
others well known in the art to produce large amounts of
polypeptide, for instance by expression from encoding nucleic
15   acid.

According to another aspect of the present invention there is
provided a nucleic acid molecule which has a nucleotide
sequence encoding a polypeptide having an amino acid sequence
20   as described above.

The coding sequence may be that of the HCV NS2/3 gene (HCV J
strain, Genbank Acc. No. D90208; HCV H strain, Genbank Acc.
No. M67463; HCV H77 strain, Genbank Acc. AF009606) or it may
25   be a mutant, variant, derivative of this sequence. The
sequence may differ from the HCV NS2/3 nucleotide sequence by
a change which is one or more of addition, insertion, deletion
and substitution of one or more nucleotides of the sequence.
Changes to a nucleotide sequence may result in an amino acid
30   change at the protein level, or not, as determined by the
genetic code.

Thus, nucleic acid according to the present invention may

include a sequence different from the nucleic acid sequence of HCV NS2/3 yet encode a polypeptide with the same amino acid sequence.

5   Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the viral genome, except

10   possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. The coding sequence shown herein is a DNA sequence. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should

15   be construed as encompassing reference to the RNA equivalent, with U substituted for T.

Nucleic acid may be provided as part of a replicable vector, and also provided by the present invention are a vector

20   including nucleic acid as set out above, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including

25   nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*.

30   Cells comprising vectors as described above are a further aspect of the present invention.

Generally, nucleic acid according to the present invention is

provided as an isolate, in isolated and/or purified form, or
free or substantially free of material with which it is
naturally associated, such as free or substantially free of
nucleic acid flanking the gene in the (e.g. viral) genome,
5 except possibly one or more regulatory sequence(s) for
expression. Nucleic acid may be wholly or partially synthetic
and may include genomic DNA, cDNA or RNA.

Nucleic acid sequences encoding the polypeptides of the
10 present invention may be readily prepared by the skilled
person using the information and references contained herein
and techniques known in the art (for example, see Sambrook,
Fritsch and Maniatis, Molecular Cloning, A Laboratory Manual,
Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al,
15 Short Protocols in Molecular Biology, John Wiley and Sons,
1992). These techniques include (i) the use of the polymerase
chain reaction (PCR) to amplify samples of such nucleic acid,
e.g. from genomic sources, (ii) chemical synthesis, or (iii)
preparing cDNA sequences. DNA encoding the polypeptides may
20 be generated and used in any suitable way known to those of
skill in the art, including taking encoding DNA, identifying
suitable restriction enzyme recognition sites either side of
the portion to be expressed, and cutting out said portion from
the DNA. The portion may then be operably linked to a
25 suitable promoter in a standard commercially available
expression system. Another recombinant approach is to amplify
the relevant portion of the DNA with suitable PCR primers.

Modifications to a nucleic acid sequence may be made, e.g.
30 using site directed mutagenesis, to lead to the production of
modified polypeptide, e.g. to take account of codon preference
in the host cells used to express the nucleic acid.

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In order to obtain expression of the nucleic acid sequences of
the invention, the sequences may be incorporated in a vector
having one or more control sequences operably linked to the
nucleic acid to control its expression. Vectors may be chosen
5   or constructed, They may contain appropriate regulatory
sequences, including promoter sequences, terminator fragments,
polyadenylation sequences, enhancer sequences, marker genes
and other sequences as appropriate, e.g. nucleic acid
sequences so that the polypeptide or peptide is produced as a
10 fusion and/or nucleic acid encoding secretion signals so that
the polypeptide produced in the host cell is secreted from the
cell. Vectors may be plasmids, viral e.g. phage, or phagemid,
as appropriate. For further details see, for example,
Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook
15 et al., 1989, Cold Spring Harbor Laboratory Press. Many known
techniques and protocols for manipulation of nucleic acid, for
example in preparation of nucleic acid constructs,
mutagenesis, sequencing, introduction of DNA into cells and
gene expression, and analysis of proteins, are described in
20 detail in Current Protocols in Molecular Biology, Ausubel et
al. eds., John Wiley & Sons, 1992.

Polypeptide may then be obtained by transforming the vectors
into host cells in which the vector is functional, culturing
25 the host cells so that the polypeptide is produced and
recovering the polypeptide from the host cells or the
surrounding medium.

A further aspect of the present invention provides a host cell
30 containing heterologous nucleic acid as disclosed herein.

Systems for cloning and expression of polypeptides in a
variety of different host cells are well known. Suitable host

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cells include bacteria, eukaryotic cells such as mammalian and
yeast, and baculovirus systems. Mammalian cell lines
available in the art for expression of a heterologous
polypeptide include Chinese hamster ovary cells, HeLa cells,
5 baby hamster kidney cells, COS cells and many others. A
common, preferred bacterial host is *E. coli*.

The nucleic acid of the invention may be integrated into the
genome (e.g. chromosome) of the host cell. Integration may be
10 promoted by inclusion of sequences which promote recombination
with the genome, in accordance with standard techniques. The
nucleic acid may be on an extra-chromosomal vector within the
cell, or otherwise identifiably heterologous or foreign to the
cell.

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A further aspect provides a method which includes introducing
a nucleic acid molecule of the invention into a host cell.
The introduction, which may (particularly for *in vitro*
introduction) be generally referred to without limitation as
20 "transformation", may employ any available technique. For
eukaryotic cells, suitable techniques may include calcium
phosphate transfection, DEAE-Dextran, electroporation,
liposome-mediated transfection and transduction using
retrovirus or other virus, e.g. vaccinia or, for insect cells,
25 baculovirus. For bacterial cells, suitable techniques may
include calcium chloride transformation, electroporation and
transfection using bacteriophage. As an alternative, direct
injection of the nucleic acid could be employed.

30 Marker genes such as antibiotic resistance or sensitivity
genes may be used in identifying clones containing nucleic
acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing
expression from the nucleic acid, e.g. by culturing host cells
(which may include cells actually transformed although more
likely the cells will be descendants of the transformed cells)
5   under conditions for expression of the gene, so that the
encoded polypeptide is produced. If the polypeptide is
expressed coupled to an appropriate signal leader peptide it
may be secreted from the cell into the culture medium.
Following production by expression, a polypeptide may be
10 isolated and/or purified from the host cell and/or culture
medium, as the case may be, and subsequently used as desired,
e.g. in the formulation of a composition which may include one
or more additional components, such as a pharmaceutical
composition which includes one or more pharmaceutically
15 acceptable excipients, vehicles or carriers (e.g. see below).

In light of the above, the present invention also provides a
method of making a truncated HCV NS2/3 protease polypeptide of
the present invention, the method including expression from
20 nucleic acid encoding the polypeptide. This may conveniently
be achieved by growing a host cell containing the nucleic acid
in culture under appropriate conditions which cause or allow
expression of the HCV NS2/3 protease polypeptide whilst
minimising auto-proteolysis. Preferred conditions include a
25 Zn-depleted or low Zn medium. This hinders the folding of the
NS3 portion of the precursor molecule, thereby impeding auto-
processing and driving the formation of insoluble protein that
accumulates in inclusion bodies.

30 Methods for the purification of insoluble proteins expressed
in inclusion bodies are well-known to persons skilled in the
art. Conveniently, gel filtration is employed under denaturing
conditions followed by reversed phase chromatography.

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The denaturated, purified protein may be renatured after
purification by adjusting the conditions to allow refolding.
This is preferably achieved in a refolding buffer in which the
chaotropic agent (guanidine) concentration has been reduced by
5   dialysis to a residual concentration of 0.75 M, ionic strength
is greater than 200mM NaCl and the concentration of protein is
less than 100ug/ml. Refolded, soluble protein can be measured
by analysing the supernatant after ultracentrifugation of the
refolded sample.
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Refolded, soluble protein may be activated by altering the
buffer conditions. A suitable buffer for activation of the
protease may include the following components:
Ionic strength equivalent to at least 50mM, preferably at
15 least 100mM, or at least 150mM NaCl, for example 200mM, 250mM
or 300mM NaCl;
10-60% glycerol, preferably 20-50% glycerol, for example 30%,
40% or 50% glycerol;
0.5%-3% CHAPS, preferably 1-2% CHAPS, for example 1%, 1.5% or
20 2% CHAPS;
pH 6.5-8.5, preferably pH 7-8, for example pH 7, 7.5 or 8;
1-100mM reducing agent (e.g. cysteine or DTT), preferably 1-
10mM reducing agent, for example, 1mM, 3mM, 5mM or 10mM
reducing agent;
25 1-100µM Zn^{++}, preferably 5-50µM Zn^{++}, for example 30µM, 40µM or
50µM Zn^{++}.

One method of activation is to reduce the protein
concentration to 2.5µg/ml or less in a buffer containing 50mM
30 TRIS pH 7.5, 50% glycerol, 2% CHAPS, 250mM NaCl, 3mM DTT, 30µM
Zn^{++} (See Example 7).

Various aspects of the present invention provide for assays

and methods of screening for and/or obtaining/identifying a
substance which modulates, e.g. inhibits, reduces or
interferes with, the activity of the HCV NS2/3 protease, and
for the use of HCV NS2/3 protease polypeptides of the present
5   invention in these screening methods and assays. The activity
of the protease may be modulated by promotion or inhibition of
dimerisation.

A screening or assay method for identifying an agent which can
10 modulate the activity of the HCV NS2/3 protease, may include:
(a)   bringing a test agent into contact with an HCV NS2/3
       protease polypeptide of the present invention; and
(b)   determining the HCV NS2/3 protease activity.

15 The HCV NS2/3 protease polypeptide which is brought into
contact with the test agent may be in the monomeric or the
dimeric form. Where the polypeptide is monomeric, the test
agent may modulate the formation of the dimeric form, the
proteolytic activity of the dimer, or both. Where the
20 polypeptide is dimeric, the test agent may modulate the
formation of the monomer form, the proteolytic activity of the
dimer, or both.

Modulation of the formation of the dimeric or monomeric form
25 may be achieved by influencing the dynamic equilibrium between
the two forms.

A screening or assay method may additionally include the step
of activating the HCV NS2/3 protease. This activation may be
30 achieved by changing the buffer conditions.

HCV NS2/3 protease polypeptides of the present invention may
form homo-dimers so an agent which affects the dimerisation of

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the polypeptide may modulate the auto-proteolytic activity.

Activity of an HCV NS2/3 protease polypeptide of the present invention may be determined by assessing the proportion of the polypeptide in the active dimeric form.

Accordingly, a screening or assay method for identifying an agent which can modulate the activity of the HCV NS2/3 protease, may include:

(a)   bringing a test agent into contact with an HCV NS2/3 protease polypeptide of the present invention; and

(b)   determining dimerisation of the HCV NS2/3 protease polypeptide.

Dimerisation may be determined by standard methods well-known to a skilled person, for example by the use of gel filtration chromatography, antibodies or the dependence of the cleavage kinetics on the protein concentration.

A related aspect of the present invention provides the use of an HCV NS2/3 protease polypeptide of the present invention for determining the presence in a test sample of an agent which has the ability to modulate the activity of the native HCV NS2/3 protease.

A method for determining the presence in a test sample of an agent which has the ability to modulate the activity of the HCV NS2/3 protease, may include:

(a)   bringing an HCV NS2/3 protease polypeptide of the present invention into contact with the test sample; and

(b)   determining activity of the HCV NS2/3 protease polypeptide.

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The method may additionally include the step of activating the HCV NS2/3 protease polypeptide. This activation may be achieved by changing the buffer conditions.

5   Activating the polypeptide may comprise promoting the formation of homo-dimers of the polypeptide.

An agent which can modulate the activity of the HCV NS2/3 protease may affect the formation of the active dimeric form.

10

A method for determining the presence in a test sample of an agent which has the ability to modulate the dimerisation of the HCV NS2/3 protease, may include:

(a) · bringing an HCV NS2/3 protease polypeptide of the present
15   invention into contact with the test sample; and

(b)   determining HCV NS2/3 protease activity.

Activity in the presence of a test substance may be compared with activity of the HCV NS2/3 protease polypeptide in
20   comparable reaction medium and conditions·in the absence of a test substance. A test substance able to modulate the activity may thereby be identified. A difference in the activity of the HCV NS2/3 protease polypeptide between the treated and untreated conditions is indicative of a modulating
25   effect of the relevant test substance(s). Activity may be related to the extent of dimerisation of the polypeptide.

The relevant test substance may achieve a modulating effect by affecting (i.e. reducing or increasing) the dimerisation of an
30   HCV NS2/3 protease polypeptide of the present invention. The presence of a modulating effect may be determined by assessing the dimerisation of the HCV NS2/3 protease polypeptide.

20

A method for determining the presence in a test sample of an
agent which has the ability to modulate the dimerisation of
the HCV NS2/3 protease, may include:

(a)   bringing an HCV NS2/3 protease polypeptide of the present
5   invention into contact with the test sample; and

(b)   determining dimerisation of the HCV NS2/3 protease
        polypeptide.

Activity may be determined by any suitable method. Examples of
10   such methods include an HPLC based approach in which activated
enzyme in incubated for a fixed time and the uncleaved
precursor separated from the products on an HPLC column, the
amount of product as measured by fluorescence being related to
the activity. Another approach involves separating the NS3
15   cleavage product from the NS2/3 precursor and the NS2 product
by means of 5' tags and quantifying the amount of NS3 by a
standard fluorimetric or radiometric assay.

A method for determining the presence in a test sample of an
20   agent which has the ability to modulate the activity of the
HCV NS2/3 protease, may include quantifying the activity of
an HCV NS2/3 protease polypeptide of the present invention
and/or the amount of said agent in the sample and/or the
amount of dimerisation of the HCV NS2/3 protease polypeptide.

25

Where appropriate, one or more controls may be included in the
assays and methods described herein. Suitable controls would
be readily employed by a skilled person.

30   Agents which modulate e.g. increase or potentiate activity of
the HCV NS2/3 protease may be identified using conditions
which, in the absence of a positively-testing agent, destroy
or reduce activity.

Methods of determining the presence of, and optionally
quantifying the amount of, an agent in a test sample which has
the ability to modulate the activity of HCV NS2/3 protease may
have a diagnostic purpose, e.g. in the evaluation of a therapy
5 to treat a condition associated with HCV infection.

A screening or assay method may include purifying and/or
isolating a test substance (e.g. an agent to be tested for the
ability to modulate HCV NS2/3 protease activity) from a
10 mixture or extract, i.e. reducing the content of at least one
component of the mixture or extract, e.g. a component with
which the test substance is naturally associated. The
screening or assay method may include determining the ability
of one or more fractions of a test mixture or extract to
15 modulate the activity of the HCV NS2/3 protease. The
purifying and/or isolating may employ any method known to
those skilled in the art.

The precise format of any of the screening or assay methods of
20 the present invention may be varied by those of skill in the
art using routine skill and knowledge. The skilled person is
well aware of the need to employ appropriate control
experiments.

25 In any assay method according to the invention, the amount of
test substance or compound which may be added to an assay of
the invention will normally be determined by trial and error
depending upon the type of compound used. Typically, from
about 0.001 nM to 1mM or more concentrations of putative
30 modulator/inhibitor compound may be used, for example from
0.01 nM to 100µM, e.g. 0.1 to 50 µM, such as about 10 µM.
Greater concentrations may be used when a peptide is the test
substance. Even a molecule which has a weak effect may be a

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useful lead compound for further investigation and
development.

A compound or agent identified by any one of the methods
5   provided by the present invention may be isolated and/or
purified and/or further investigated and/or manufactured.
Various methods and uses of such compounds are discussed
elsewhere herein. The present invention thus provides methods
of identifying agents which have the ability to modulate the
10   activity of HCV NS2/3 protease.

An agent or substance employed in a method in accordance with
the present invention may be a natural or synthetic chemical
compound and may be an organic, inorganic, peptide, nucleic
15   acid or other molecule. Suitable compounds which may be
screened include natural or synthetic chemical compounds used
in drug screening programmes. Extracts of plants, microbes or
other organisms, which contain several characterised or
uncharacterised components may also be used.
20

It is worth noting that combinatorial library technology
provides an efficient way of testing a potentially vast number
of different substances for ability to modulate an
interaction. Such libraries and their use are known in the
25   art, for all manner of natural products, small molecules and
peptides, among others. The use of peptide libraries may be
preferred in certain circumstances.

One class of putative modulators comprises peptide fragments
30   derived from HCV NS2/3 protease polypeptides of the present
invention, or alleles, mutants or derivatives of such
fragments. Peptide fragments of from 5 to 40 amino acids, for
example from 6 to 10 amino acids from the regions of the

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polypeptide where cleavage occurs or which are responsible for activity, may be tested for their ability to inhibit auto-proteolysis.

5   Other suitable peptides are those which modulate the activity of an HCV NS2/3 protease polypeptide of the present invention and which have a length of 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100, or more than 100 amino acids.

10

Other suitable peptides are those which inhibit the dimerisation of an HCV NS2/3 protease polypeptide of the present invention and which have a length of 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100, or more

15 than 100 amino acids.

Nucleic acid encoding such fragments, vectors and host cells containing such nucleic acid, and methods of expressing nucleic acid encoding such fragments are further aspects of

20 the present invention.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of the HCV NS2/3 protease polypeptide and using rational drug design to provide

25 potential inhibitor compounds with particular molecular shape, size and charge characteristics.

Following identification of a substance which modulates   or affects protease activity, the substance may be investigated

30 further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

24

In various aspects, the present invention provides a modulator
identified by a screening method of the invention, e.g. a
substance which inhibits or diminishes, increases or
potentiates activity of the HCV NS2/3 protease.

5

The modulator may be purified and/or investigated further
and/or manufactured, following identification. A modulator may
be used to obtain peptidyl or non-peptidyl mimetics, e.g. by
methods well known to those skilled in the art and discussed

10   herein. It may be used in a therapeutic context as discussed
below.

Antibodies directed to an HCV NS2/3 protease polypeptide of
the present invention form a further class of putative

15   inhibitor compounds. Candidate inhibitor antibodies may be
characterised and their binding regions determined to provide
single chain antibodies and fragments thereof which are
responsible for affecting the protease activity.

20   Antibodies may be obtained using techniques which are standard
in the art. Methods of producing antibodies include
immunising a mammal (e.g. mouse, rat, rabbit, horse, goat,
sheep or monkey) with an HCV NS2/3 protease polypeptide of the
present invention. Antibodies may be obtained from immunised

25   animals using any of a variety of techniques known in the art,
and screened for modulation of HCV NS2/3 activity using an HCV
NS2/3 protease polypeptide of the present invention. For
instance, Western blotting techniques or immunoprecipitation
may be used (Armitage *et al.*, 1992, Nature **357**: 80-82).

30   Isolation of antibodies and/or antibody-producing cells from
an animal may be accompanied by a step of sacrificing the
animal.

25

As an alternative or supplement to immunising a mammal with a
peptide, an antibody specific for an HCV NS2/3 protease
polypeptide of the present invention may be obtained from a
recombinantly produced library of expressed immunoglobulin
5 variable domains, e.g. using lambda bacteriophage or
filamentous bacteriophage which display functional
immunoglobulin binding domains on their surfaces; for instance
see WO92/01047. The library may be naive, that is constructed
from sequences obtained from an organism which has not been
10 immunised with an HCV NS2/3 protease polypeptide of the
present invention (or fragments thereof), or may be one
constructed using sequences obtained from an organism which
has been exposed to the antigen of interest. Candidate
antibodies may be screened for modulation of HCV NS2/3
15 activity using the NS2/3 protease polypeptide.

Antibodies according to the present invention may be modified
in a number of ways. Indeed the term "antibody" should be
construed as covering any binding substance having a binding
20 domain with the required specificity. Thus the invention
covers antibody fragments, derivatives, functional equivalents
and homologues of antibodies, including synthetic molecules
and molecules whose shape mimicks that of an antibody enabling
it to bind an antigen or epitope.

25

Example antibody fragments, capable of binding an antigen or
other binding partner are the Fab fragment consisting of the
VL, VH, Cl and CH1 domains; the Fd fragment consisting of the
VH and CH1 domains; the Fv fragment consisting of the VL and
30 VH domains of a single arm of an antibody; the dAb fragment
which consists of a VH domain; isolated CDR regions and
F(ab')2 fragments, a bivalent fragment including two Fab
fragments linked by a disulphide bridge at the hinge region.

Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the
present invention may be subject to genetic mutation or other
5 changes. It will further be understood by those skilled in
the art that a monoclonal antibody can be subjected to the
techniques of recombinant DNA technology to produce other
antibodies or chimeric molecules which retain the specificity
of the original antibody. Such techniques may involve
10 introducing DNA encoding the immunoglobulin variable region,
or the complementarity determining regions (CDRs), of an
antibody to the constant regions, or constant regions plus
framework regions, of a different immunoglobulin. See, for
instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and
15 expression of chimeric antibodies are described in EP-A-
0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding
characteristics are within the scope of the present invention,
20 as are host cells, eukaryotic or prokaryotic, containing
nucleic acid encoding antibodies (including antibody
fragments) and capable of their expression. The invention
also provides methods of production of the antibodies
including growing a cell capable of producing the antibody
25 under conditions in which the antibody is produced, and
preferably secreted.

Antibodies may also be used in purifying and/or isolating HCV
NS2/3 protease polypeptides of the present invention, for
30 instance, following production of the polypeptide by
expression from encoding nucleic acid therefor. Antibodies
may be useful in a therapeutic context (which may include
prophylaxis) to disrupt activity of the HCV NS2/3 protease

with a view to inhibiting the replication of HCV and thereby reducing or preventing HCV infection. Antibodies can for instance be micro-injected into cells or tissues or administered systemically. Antibodies may be employed in

5 accordance with the present invention for other therapeutic and non-therapeutic purposes which are discussed elsewhere herein.

In a further aspect, the present invention provides the use of

10 an agent which is capable of modulating the activity of an HCV NS2/3 protease polypeptide of the present invention, in a method of designing a peptide or non-peptidyl mimetic of the compound, which mimetic is able to modulate the activity of HCV NS2/3 protease. The agent used in such a method may be an

15 agent identified using methods according to the present invention.

The present invention similarly provides for the use of an HCV NS2/3 protease polypeptide of the present invention in a

20 method of designing a peptide or non-peptidyl mimetic of a HCV NS2/3 protease, which mimetic is able to modulate the activity of the HCV NS2/3 protease.

Accordingly, the present invention provides a method of

25 designing a mimetic of a compound which has the biological activity of modulating the activity of HCV NS2/3 protease, as determined by methods of the present invention, or a method of designing a mimetic of an HCV NS2/3 protease polypeptide of the present invention which has the biological activity of

30 modulating the activity of HCV NS2/3 protease, as determined by methods of the present invention, said method comprising:
        (i)   analysing a substance having the biological activity to determine the amino acid residues essential and important

for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

5   Suitable modelling techniques are known in the art. Such techniques enable the study of the interaction between an HCV NS2/3 protease polypeptide of the present invention and a modulating compound and to design compounds which contain functional groups arranged in such a manner that they could

10   reproduce that interaction.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be

15   desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, for instance compounds of the present invention that are peptides may not be well suited as active agents for oral compositions as they tend to be quickly

20   degraded by proteases in the alimentary canal.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are

25   critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are

30   known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g.

29

stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a

5 pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of the above approach, the three-dimensional structure of a ligand and its binding partner are modelled.

10 This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical

15 groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological

20 activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or

25 clinical testing.

The mimetic or mimetics found by any of the approaches described herein may be used in the assay methods of the present invention to determine whether they have the ability

30 to modulate the activity of the HCV NS2/3 protease.

Mimetics obtained by a method of the invention form a further aspect of the invention.

As used herein, a variant of a stated amino acid sequence may
differ by one or more amino acid residues from that sequence,
by one or more of addition, insertion, deletion and
substitution of one or more amino acid residues. It may ›
5   include 1, 2, 3, 4, 5, or greater than 5 amino acid
alterations such as substitutions with respect to the stated
sequence.

Such a variant of an HCV NS2/3 protease polypeptide of the
10 present invention, which has a sequence disclosed herein, may,
in certain embodiments, be the same length or shorter than
that sequence. In other embodiments, the HCV NS2/3 protease
polypeptide of the present invention (or a variant thereof)
may be included in a larger polypeptide particularly where the
15 HCV NS2/3 protease polypeptide is fused to a heterologous or
foreign sequence. For example, 1, 2, 3, 4 or 5, 10, 20 or
more additional amino acid residues, heterologous to a native
form of the specific HCV NS2/3 protease polypeptide, may be
included at one end or both ends of the HCV NS2/3 protease
20 polypeptide.

Derivatives of polypeptides include the polypeptide linked to
a coupling partner, e.g. an effector molecule, a label, a
drug, a toxin and/or a carrier or transport molecule, and/or a
25 targeting molecule such as an antibody or binding fragment
thereof or other ligand. Techniques for coupling to both
peptidyl and non-peptidyl coupling partners are well known in
the art. In one embodiment, the carrier molecule is a 16
amino acid peptide sequence derived from the homeodomain of
30 *Antennapedia* (e.g. as sold under the name "Penetratin"), which
can be coupled to a peptide via a terminal Cys residue. The
"Penetratin" molecule and its properties are described in WO
91/18981.

HCV NS2/3 protease polypeptides of the present invention
and/or peptide modulating agents may be generated wholly or
partly by chemical synthesis, in accordance with well-
5   established techniques, such as standard liquid or,
preferably, solid-phase peptide synthesis methods, general
descriptions of which are broadly available (see, for example,
in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis,
2nd edition, Pierce Chemical Company, Rockford, Illinois
10 (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of
Peptide Synthesis, Springer Verlag, New York (1984); and
Applied Biosystems 430A Users Manual, ABI Inc., Foster City,
California), or they may be prepared in solution, by the
liquid phase method or by any combination of solid-phase,
15 liquid phase and solution chemistry.

The invention further provides various therapeutic methods and
uses of one or more substances selected from (i) a compound
identified in accordance with a method of the invention which
20 is able to modulate the activity of the HCV NS2/3 protease;
(ii) a mimetic of any of the above substances which is able to
modulate the activity of the HCV NS2/3 protease.

The therapeutic/prophylactic purpose of such a method or use
25 may be the modulation, e.g. disruption or interference, of the
activity of the HCV NS2/3 protease, thereby to disrupt the
replication of HCV and hence reduce or prevent HCV infection.

In various further aspects the present invention thus provides
30 a pharmaceutical composition, medicament, drug or other
composition for such a purpose, the composition comprising one
or more of those substances, the use of such a substance in a
method of medical treatment, a method comprising

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administration of such a substance or composition to a
patient, e.g. for treatment(which may include preventative
treatment) of a medical condition, e.g. a condition associated
with HCV infection, use of such a substance in the manufacture
5   of a composition, medicament or drug for administration for
such a purpose, e.g.for treatment of a condition associated
with HCV infection, and a method of making a pharmaceutical
composition comprising admixing such a substance with a
pharmaceutically acceptable excipient, vehicle or carrier, and
10 optionally other ingredients.

The substances may be used as sole active agents or in
combination with one another or with any other active
substance.

15

Whatever the substance used in a method of medical treatment
of the present invention, administration is preferably in a
"prophylactically effective amount" or a "therapeutically
effective amount" (as the case may be, although prophylaxis
20 may be considered therapy), this being sufficient to show
benefit to the individual. The actual amount administered,
and rate and time-course of administration, will depend on the
nature and severity of what is being treated. Prescription of
treatment, e.g. decisions on dosage etc, is within the
25 responsibility of general practitioners and other medical
doctors.

A substance or composition may be administered alone or in
combination with other treatments, either simultaneously or
30 sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present
invention, and for use in accordance with the present

invention, may include, in addition to active ingredient, a
pharmaceutically acceptable excipient, carrier, buffer,
stabiliser or other materials well known to those skilled in
the art. Such materials should be non-toxic and should not
5   interfere with the efficacy of the active ingredient. The
precise nature of the carrier or other material will depend on
the route of administration, which may be oral, or by
injection, e.g. cutaneous, subcutaneous or intravenous.

10 Pharmaceutical compositions for oral administration may be in
tablet, capsule, powder or liquid form. A tablet may include
a solid carrier such as gelatin or an adjuvant. Liquid
pharmaceutical compositions generally include a liquid carrier
such as water, petroleum, animal or vegetable oils, mineral
15 oil or synthetic oil. Physiological saline solution, dextrose
or other saccharide solution or glycols such as ethylene
glycol, propylene glycol or polyethylene glycol may be
included.

20 For intravenous, cutaneous or subcutaneous injection, or
injection at the site of affliction, the active ingredient
will be in the form of a parenterally acceptable aqueous
solution which is pyrogen-free and has suitable pH,
isotonicity and stability. Those of relevant skill in the art
25 are well able to prepare suitable solutions using, for
example, isotonic vehicles such as Sodium Chloride Injection,
Ringer's Injection, Lactated Ringer's Injection.
Preservatives, stabilisers, buffers, antioxidants and/or other
additives may be included, as required.

30
Liposomes, particularly cationic liposomes, may be used in
carrier formulations.

34

Examples of techniques and protocols mentioned above can be
found in Remington's Pharmaceutical Sciences, 16th edition,
Osol, A. (ed), 1980.

5   The substance or composition may be administered in a
    localised manner to a desired site or may be delivered in a
    manner in which it targets particular cells.

    Targeting therapies may be used to deliver the active
10 substance more specifically to certain types of cell, by the
    use of targeting systems such as antibody or cell specific
    ligands. Targeting may be desirable for a variety of reasons,
    for example if the agent is unacceptably toxic, or if it would
    otherwise require too high a dosage, or if it would not
15 otherwise be able to enter the target cells.

    Instead of administering such substances directly, they may,
    where the modulating substances are polypeptides, be produced
    in the target cells by expression from an encoding nucleic
20 acid introduced into the cells, e.g. from a viral vector. The
    vector may be targeted to the specific cells to be treated, or
    it may contain regulatory elements which are switched on more
    or less selectively by the target cells.

25 Nucleic acid encoding the substance e.g. a polypeptide able to
    modulate the activity of the HCV NS2/3 protease may thus be
    used in methods of gene therapy, for instance in treatment of
    individuals, e.g. with the aim of preventing or curing (wholly
    or partially) a disorder associated with HCV infection.
30
    Vectors such as viral vectors have been used in the prior art
    to introduce nucleic acid into a wide variety of different
    target cells. Typically the vectors are exposed to the target

35

cells so that transfection can take place in a sufficient
proportion of the cells to provide a useful therapeutic or
prophylactic effect from the expression of the desired
polypeptide. The transfected nucleic acid may be permanently
5 incorporated into the genome of each of the targeted cells,
providing long lasting effect, or alternatively the treatment
may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors,
10 are known in the art, see US Patent No. 5,252,479 and WO
93/07282. In particular, a number of viruses have been used
as gene transfer vectors, including papovaviruses, such as
SV40, vaccinia virus, herpesviruses, including HSV and EBV,
and retroviruses. Many gene therapy protocols in the prior
15 art have used disabled murine retroviruses.

As an alternative to the use of viral vectors in gene therapy
other known methods of introducing nucleic acid into cells
includes mechanical techniques such as micro-injection,
20 transfer mediated by liposomes and receptor-mediated DNA
transfer.

Receptor-mediated gene transfer, in which the nucleic acid is
linked to a protein ligand via polylysine, with the ligand
25 being specific for a receptor present on the surface of the
target cells, is an example of a technique for specifically
targeting nucleic acid to particular cells.

An agent or substance having an ability to modulate the
30 activity of the HCV NS2/3 protease or a nucleic acid molecule
which encodes a polypeptide having that ability, may be
provided in a kit, e.g. sealed in a suitable container which
protects its contents from the external environment. Such a

kit may include instructions for use.

In still further aspects the present invention provides for
the purification of a polypeptide, protein or other substance
5 which has the ability to modulate the activity of the HCV
NS2/3 protease. The invention also provides for a purified
protein, polypeptide or other substance which has the ability
to modulate the activity of the HCV NS2/3 protease. The
purified protein, polypeptide or other substance may be about
10 10% pure, more preferably about 20% pure, more preferably
about 30% pure, more preferably about 40% pure, more
preferably about 50% pure, more preferably about 60% pure,
more preferably about 70% pure, more preferably about 80%
pure, more preferably about 90% pure, more preferably about
15 95% pure, or substantially pure.

In another aspect, the present invention provides a method of
purifying a protein, .polypeptide or other substance which has
the ability to modulate the activity of the HCV NS2/3
20 protease, the method including contacting the protein,
polypeptide or other substance with an HCV NS2/3 protease
polypeptide of the present invention.

A mixture of material including a protein, polypeptide or
25 other substance which has the ability to modulate the activity
of the HCV NS2/3 protease may be contacted against immobilised
an HCV NS2/3 protease polypeptide of the present invention
(e.g. immobilised either covalently or non-covalently such as
via a specific binding molecule such as streptavidin or
30 biotin) and molecules which do not bind to the polypeptide are
washed off.

The protein, polypeptide or other substance which has the

ability to modulate the activity of the HCV NS2/3 protease, in
a purification method of the present invention, may be in a
mixture of molecules, such as a cellular extract, such as a
normal cell of an organism such as a human or a recombinant
5   host cell expressing the protein or polypeptide or its
unphosphorylated form from encoding DNA, such as a bacterial,
eukaryotic (e.g. mammalian or yeast) or insect cell, such as
in a baculovirus expression system.

10 Following purification, the protein, polypeptide or other
substance which has the ability to modulate the activity of
the HCV NS2/3 protease may be used as desired, e.g. in a
therapeutic context.

15 Figure 1 shows a scheme for a high throughput screening assay
for the NS2/3 protease inhibitors. In step 1, NS2/3 is
refolded. The precursor is then activated in an appropriate
buffer so that cleavage occurs (step 2). Uncleaved precursor
is then captured (step 4) and the amount of free NS3 in
20 solution measured (step 5).

Figure 2 shows the cleavage reaction products of NS2/3
seperated by HPLC, as follows. A solution of 5 μM
H_6-907-1206-ASK4 protease in 6 M guanidine hydrochloride, 25
25 mM Tris pH 8.7, 100 mM DTT were diluted 50-fold into a buffer
containing 50 mM Tris pH 7.5, 3 mM DTT, 50% glycerol, 1%
CHAPS, 50 μM $ZnCl_2$, 250 mM NaCl at a temperature of 4°C. After
5 minutes the temperature was raised to 23°C thereby
initiating the cleavage reaction. Samples were analysed on a
30 Poros R1/H perfusion chromatography column (4.6 mm x 50 mm)
equilibrated with 90% H20/0.1%TFA (buffer A) and 10%
acetonitrile/0.08% TFA (buffer B). The column was operated at
a flow rate of 2.5 ml/min using a Merck-Hitachi high

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performance liquid chromatograph equipped with a fluorescence
detector. A gradient from 10% - 90% B in 15 minutes was used
to separate the precursor from its cleavage fragments. Protein
peaks were detected by monitoring of tryptophan fluorescence

5   (excitation 280 nm, emission 350 nm) and quantified by peak
integration. Top gradient is refolded NS2/3, middle gradient
is activated NS2/3 O.N. and bottom gradient is purified NS3.

Figure 3 shows an analysis of the cleavage reaction of 300 nM

10 H_6-907-1206-ASK4 protease by HPLC. Left panel: analysis of the
time course of the NS2/3 cleavage reaction. The area of the
HPLC peak corresponding to the NS3 cleavage product was
determined by peak integration and plotted as a function of
incubation time. Data could be fitted with a single

15 exponential equation to derive a value for the observed first
order rate contant of the reaction. Right panel: Concentration
dependence of NS2/3 self cleavage at 23°C. The first order
rate constants of the cleavage reaction were determined as a
function of protein concentration.

20

Figure 4 shows a diagram of two expression plasmid vectors,
pT7.7 (upper plasmid) and pCITE 2b (+) (lower plasmid) used
for the cloning of fragments of cDNA encoding for the NS2/3
region of the HCV polyprotein.

25

EXPERIMENTAL
EXAMPLE 1
Subcloning of the NS2/3 protease in expression vectors

30 PCR amplification of NS2-3 constructs
Plasmids suitable for the heterologous expression of active,
wild type, or inactive mutants of NS2/3 have been generated by
cloning of PCR-amplified fragments of cDNA encoding for the

NS2/3 region of the HCV polyprotein into appropriate restriction sites.

Several expression plasmids are known in the art, both for
5 eukaryotic and prokaryotic heterologous expression of proteins. In the present example the backbone of the pT7.7 vector is used for heterologous expression in prokaryotic (E. coli) cells, while pCITE 2b (+) is used for heterologous expression in eukaryotic cells (Hep 3b) or *in vitro*
10 transcription/translation system. In principle other expression systems could be used to the same purpose.

cDNAs encoding the non-structural region of isolates corresponding to HCV strains J or H were used as templates for
15 PCR amplification (HCV J strain, Genbank Acc. No. D90208; HCV H strain, Genbank Acc. No. M67463; HCV H77 strain, Genbank Acc. AF009606). DNA was routinely amplified by preparing an amplification reaction mixture composed of 20 U/ml Taq DNA polymerase mix in 60 mM Tris-SO$_4$ (pH 9.1 at 25 C), 18 mM
20 (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 200 µM dTTP and stabilisers (PCR SuperMix High Fidelity, GibcoBRL, Cat. No.: 10790-020), called PCR protocol 1 in the following examples.

25 Alternatively, DNA was amplified by preparing an amplification reaction mixture composed of 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, pH 8.3 (20 C), 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 200 µM dTTP and 2.5 U of Taq DNA Polymerase (*Taq* DNA Polymerase, Boehringer Mannheim, Cat. No.: 1146 173), called
30 PCR protocol 2 in the following examples.

In some cases, a different amplification reaction mixture was used, composed of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM

MgCl2, 0.001% gelatin, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 200 µM dTTP and 2.5 U of AmpliTaq Gold(tm) (Perkin Elmer, Cat. No.: N808-0241), called PCR method 3 in the following examples.

In all cases, the reaction volume was 25 µl, specific primers were 200-500 nM each (final concentration) and template DNA was routinely between 20 and 100 ng (total amount per reaction). Reactions were assembled on ice, mixed thoroughly and loaded on the thermocycler at 95°C. PCR amplification was performed for 20-30 cycles of 95°C, 60 seconds. Annealing was generally performed for 15 seconds at a temperature 5°C lower than the lowest melting temperature (Tm value) of the primers. Methods to calculate Tm values from the oligonucleotide sequence are known in the art and can be found in Sambrook et al. (1989). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press.

Extension was performed at 72°C (Taq DNA Polymerase, Boehringer Mannheim) or 68°C (PCR SuperMix High Fidelity, GibcoBRL) for 60 second/Kb target length.

All PCR reactions were preceded by a single 1-2 minutes denaturation cycle performed at 95°C (or 7 minutes for reactions performed with Taq Gold) and were terminated by a quick ramp to 4°C, alternatively the ramp to 4°C was preceded by a single cycle performed at 72°C for 7 minutes (see below for specific conditions). The thermocycler was from Perkin Elmer (GeneAMP PCR System 9700).

Following is a list of the oligonucleotide primers used for amplification:

41

Primer	Name	Sequence (5' to 3')
1	H NS3 Rev ASK4	ttggaattcctacttcttcttcttgctagctctcatg gttgtctctaggttctc
2	J NS2 Forw H6	ggccacccaccaccaccaccaccacatggaccgagag atggctgcatcg
3	J NS3 Rev ASK4 bis	ctacttcttcttcttgctagcccgcatggtagtttcc atagactc
4	J NS2 Forw	accgagagatggctgcatcgtgcgg
5	J NS2 (903) Forw	caggctggcatgactagagtgccg
6	J NS2 (907) Forw	actagagtgccgtactttgtacgc
7	J NS2 (913) Forw	tacgcgctcaggggctcatccgtgc
8	J NS2 (919) Forw	atccgtgcatgcatgttagtgcgg
9	J NS2 (919) H6 Forw	caccaccaccaccacatccgtgcatgcatgttag tgcgg
10	J NS2 Rev	ctaaaggagccgccacccctgtagacc

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10	J NS2 Rev	ctaaaggagccgccacccctgtagacc
11	J NS3 Rev	ctaccgcatggtagtttccatagactc
12	J 903 pT7H6	tgcatcatcatcatcatcatcaggctggcatgactag agtgccg
13	J 903 pT7	tgcaggctggcatgactagagtgccg
14	J 907 pT7H6	tgcatcatcatcatcatcatactagagtgccgtactt tgtacgc
15	J 907 pT7	tgactagagtgccgtactttgtacgc
16	H NS2 Forw H6	ccacccaccaccaccaccaccacctggacacggaggt ggccgcgtcg

Mutagenesis

Mutations were generally introduced by PCR-amplification of
cDNA sequences using mutagenic primers. This method is well

5   known to those skilled in the art and is described in:
Sambrook *et al.* (1989), Molecular cloning, a laboratory
manual. Cold Spring Harbor Laboratory Press, Ehrlich, H. A.,
(1989) PCR Technology Stockton Press, New York. and Zhao *et
al.*(1993) Methods in Enzymology 217, 218. The PCR conditions

10 were those described above.

Mutations can also be introduced using the U.S.E. (Unique Site
Elimination) method of Deng, W.P. and Nickoloff, J.A., (1992)
Anal. Biochem. 200, 81, with the U.S.E. Mutagenesis kit

15 (Pharmacia Biotech, Cat. No.: 27-1699-01) or by restriction

43

enzyme-subcloning of fragments containing the desired mutation(s).

Following is a list of the primers used for mutagenesis:

Primer	Name	Sequence (5' to 3')
17	H77 Forw (A1027P)	aggttgctgccgcccatcacggcg
18	H77 Rev (A1027P)	cgccgtgatgggcggcagcaacct
19	H77 Forw (H952A)	gtgtataacgctctcacccctctgc
20	H77 Rev (H952A)	gcagaggggtgagagcgttatacac
21	J (H952A) Forw Bis	acgtacgtatatgacgctcttactccactgc gg
22	J (H952A) Rev Bis	ccgcagtggagtaagagcgtcatatacgtac gt
23	J Forw (A1027P)	cggctccttccgcctatcacggcc
24	J Rev (A1027P)	ggccgtgataggcggaaggagccg
25	J (C993A) Forw Not I/Eag I	gcagacaccgcggccgctggggacatcatc
26	J (NdeI -) Forw	ggctcatctggtggttacaatattttatcac cagagccg

		cagagccg
27	U.S.E. Selection Primer Sca I/Mlu I	ctgtgactggtgacccgtcaaccaagtc

<u>Subcloning of NS2-3 in pT7.7</u>

pT7.7 was the vector of choice in preparing constructs to be used for heterologous protein expression in prokaryotes (Studier, F.W., Rosenberg, A.H., Dunn, J.J. &Dubendorff, J.W. 1989. Methods in Enzymology 185, 60) CsCl$_2$ quality DNA (prepared according to Sambrook *et al* 1989: Ref above) was restricted with Nde I (Boehringer Mannheim) for 3 hrs at 37°C and filled-in with Klenow DNA Polymerase (Boehringer Mannheim) following manufacturer instructions, i.e. by adding 33 µM of NTP and 1 U of enzyme / µg of plasmid for fifteen minutes at 25°C. The filled-in plasmid was loaded on a 1% TAE agarose gel, stained with 5 µg/ml ethidium bromide and electrophoresed.

After electrophoresis, the band corresponding to plasmid DNA was excised and gel purified with the Quiaex II kit (Quiagen, Cat No.: 20021). Following elution, the plasmid was dephosphorylated with Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim) following manufacturer instructions and solution purified with the Quiaex II kit (Quiagen, Cat No.: 20021).

Inserts derived from PCR protocol 1 were phosphorylated directly in the PCR mix by addition of 5 mM ATP and 7.9U of T4 Polynucleotide Kinase (Pharmacia, Cat. No.: 27-0736-02) for 30 minutes at 37°C. Following phosphorylation, PCR products were loaded on a 1% TAE agarose gel, stained with 5 µg/ml ethidium

45

bromide and electrophoresed. After electrophoresis the bands
of interest were excised from the gel and eluted with the
Quiaex II kit.

5   Ligations were performed by mixing the plasmid with the insert
in a 1:3 molar ratio in a ligation mixture composed of 50 mM
Tris-HCl (pH 8.7), 10 mM DTT, 1 mM ATP and 25 µg/ml bovine
serum albumin with 400U of T4 DNA Ligase (New England Biolabs,
Cat. No.: 202S) at 16°C for at least 1 hour; alternatively the
10 Rapid DNA Ligation kit was used following manufacturer
instructions (Boehringer Mannheim, Cat. No.: 1635 379). Where
possible mutations where shuttled from one construct to the
other by restriction.

15 Following is a list of the constructs prepared in pT7.7 and
used throughout these examples, [cloning site is Nde I for all
pT7.7 constructs]:

Const No	Name	PCR method	5' primer	3' primer	mutagenic primer
1	pT7-7 J H6-903-1206-K4 wt	1	12	3	/
2	pT7-7 J 903-1206 wt	1	13	11	/
3	pT7-7 J H6-907-1206-K4 wt	1	14	3	/
	pT7-7J				

4	H6-907-1206-K4 (H952A)	1	14	3	21, 22
5	pT7-7JH6-907-1206-K4 (A1027P)	1	14	3	23, 24
6	pT7-7 JH6-907-1206-K4 . (C993A)	1	14	3	25, 27
7	pT7-7 J 907-1206-K4 wt	1	15	3	/
8	pT7-7 J 907-1206-K4 (H952A)	1	15	3	21, 22
9	pT7-7 J 907-1206-K4 (C993A)	1	15	3	25, 27
10	pT7-7 J 907-1206-K4 (A1027P)	1	15	3	23, 24
11	pT7-7 J 907-1206 wt	1	15	11	/
12	pT7-7 J 907-1206(H952A)	1	15	11	21, 22

13	pT7-7 J 907-1206(A1027P)	1	15	11	23, 24
14	pT7-7 J H6-907-1026 wt	1	14	11	/
15	pT7-7 J 907-1026 wt	1	15	11	/

Subcloning of NS2-3 in pCITE 2b (+)

pCITE 2b (+) (Novagen, Cat. No.: 69291-1) was the vector of
choice for preparing constructs to be used for eukaryotic
expression experiments or in vitro translation assays. CsCl₂
quality DNA was restricted with Nco I for 3 hrs at 37°C and
filled-in with Klenow DNA Polymerase following manufacturer
instructions, i.e. by adding 33 µM of NTP and 1 U of enzyme/µg
of plasmid for fifteen minutes at 25°C. The filled-in plasmid
was loaded on a 1% TAE agarose gel, stained with 5 µg/ml
ethidium bromide and electrophoresed. After electrophoresis
the band corresponding to the plasmid DNA was excised and gel
purified with the Quiaex II kit. Following elution, plasmid
DNA was dephosphorylated with calf intestinal alkaline
phosphatase following manufacturer instructions and solution
purified.

Alternatively, the plasmid was restricted with MluN I and
dephosphorylated following the protocol outlined above. In
some other cases, pCITE 2b (+) was restricted with MluN I,
EcoR I and dephosphorylated as above.

Inserts derived from PCR protocol 1 and 2 were phosphorylated
directly in the PCR mix by addition of 5 mM ATP and 7.9 U of

T4 Polynucleotide Kinase for 30 minutes at 37°C. Following
phosphorylation, PCR products were loaded on a 1% TAE agarose
gel, stained with 5 µg/ml ethidium bromide and
electrophoresed.

After electrophoresis the band of interest was excised from
the gel and eluted with the Quiaex II kit. When inserts were
derived from PCR protocol 3, the cloning strategy implicated a
first cloning step in pCRII.I (Invitrogen, Cat. No.:
K2000-01), cloned fragments were then rescued by MluN I, EcoR
I restriction and subcloned in pCITE 2b (+). When required by
the cloning strategy, inserts prepared with PCR protocol 2 or
3 were polished with T4 DNA Polymerase (New England Biolabs,
Cat. No.: 203S) according to manufacturer instructions and
purified as outlined previously.

Ligations were performed by mixing the plasmid with the insert
in a 1:3 molar ratio in a ligation mixture composed of 50 mM
Tris-HCl (pH 8.7), 10 mM DTT, 1 mM ATP and 25 µg/ml bovine
serum albumin with 400U of T4 DNA at 16°C for at least 1 hour;
alternatively the Rapid DNA Ligation kit was used following
manufacturer instructions.

Where possible, mutations where shuttled from one construct to
the other by restriction.

Following is a list of the constructs prepared in pCITE 2b
(+):

Con s No	Name	Clonin g site	PCR Metho d	5' prime r	3' prime r	mutagen ic primer

16	pCITE 2b (+) H H6-810-1206-K4 wt	MluN I EcoR I	2, 3	16	1	/
17	pCITE 2b (+) H H6-810-1206-K4 (H952A)	MluN I EcoR I	2, 3	16	1	19, 20
18	pCITE 2b (+) H H6-810-1206-K4 (A1027P)	MluN I EcoR I	2, 3	16	1	17, 18
19	pCITE 2b (+) J H6-811-1206-K4 wt	MluN I EcoR I	2, 3	2	3	/
20	pCITE 2b (+) J H6-811-1206-K4 (H952A)	MluN I EcoR I	2, 3	2	3	19, 20
21	pCITE 2b (+) J H6-811-1206-K4 (A1027P)	MluN I	2, 3	2	3	17, 19
22	pCITE 2b (+) J 810-1206 wt	MluN I	1	4	11	26
23	pCITE2b(+)J 810-1206 (H952A)	MluN I	1	4	11	19, 20, 26

24	pCITE 2b (+) J 810-1206 (A1027P)	MluN I	1	4	11	17, 18, 26
25	pCITE 2b (+) J 903-1206 wt	Nco I	1	5	11	/
26	pCITE 2b (+) J 903-1206 (H952A)	Nco I	1	5	11	19, 20
27	pCITE 2b (+) J 903-1206 (A1027P)	Nco I	1	5	11	17, 18
28	pCITE 2b (+) J 907-1206 wt	Nco I	1	6	11	/
29	pCITE 2b (+) J 907-1206 (H952A)	Nco I	1	6	11	19 , 20
30	pCITE 2b (+) J 907-1206 (A1027P)	Nco I	1	6	11	17, 18
31	pCITE 2b (+) J 913-1206 wt	MluN I	1	7	11	/

32	pCITE 2b (+) J 919-1206 wt	Nco I	1	8	11	/
33	pCITE 2b (+) J H6-919-1206 wt	Nco I	1	9	11	/
34	pCITE 2b (+) J 810-1026 wt	MluN I	1	4	11	26
35	pCITE 2b (+) J 810-1026 (H952A)	MluN I	1	4	11	19, 20, 26

Transformation of competent cells

For cloning purposes and DNA propagation all constructs were
transformed in E. coli Top10 cells; transformation was
accomplished according to the CaCl$_2$ method Sambrook et al
(Sambrook, J., Fritsch E,.F., Maniatis, T., 1989, Molecular
cloning, a laboratory manual. Cold Spring Harbor Laboratory
Press.)

All deletion mutants were generated in the context of the HCV
J-strain and then cloned into a pCITE vector and tested in a
coupled *in vitro* transcription/translation system (see below).
The deletion mutants starting at residues 903 and 907 showed
full cleavage activity when compared with a wild-type
construct, starting at residue 810, whereas NS2/3 913-1207 was
inactive.

All constructs yielded high levels (> 5 mg/l) of heterologous
protein expression in *E. coli*. A high level of auto-
processing, varying from 50 to 80%, was observed after 3 hours
induction at 23°C.

EXAMPLE 2

Expression of NS2/3 protease constructs in *in vitro*
transcription/translation systems (IVT).

5

In vitro translation (IVT) assays were performed with the
Rabbit Reticulocyte Lysate System or with the TnT T7 Quick
Coupled Transcription/Translation System (Promega, Cat. No.:
L4151 and L1170) following manufacturer instructions.

10

DNA suitable for IVT was prepared by $CsCl_2$ or with the Wizard
Plus SV DNA Purification System (Promega, Cat. No.: A1460).
DNA was generally linearized with the appropriate restriction
enzyme for 3 hrs and precipitated with CH_3COONa/EtOh. In some

15   cases, DNA was phenol extracted from solution before EtOh
precipitation. Precipitated DNA was then resuspended in water
and a small aliquot loaded on a 1% TAE agarose gel stained
with ethidium bromide, to check restriction and concentration.
Linear DNA was used as a template for *in vitro* T7 RNA

20   polymerase-driven transcription (Stratagene, Cat. No.:
600124).

Transcribed RNA was phenol extracted and precipitated with
CH_3COONa /EtOh. RNA was then separated from template DNA by

25   centrifugation through a G-50 spin column (Pharmacia, Cat.
No.:17-0043-01) (Sambrook, J., Fritsch E,.F., Maniatis, T.,
(1989). Molecular cloning, a laboratory manual. Cold Spring
Harbor Laboratory Press.) Purified RNA was precipitated as
above and resuspended in water.

30

RNA was routinely translated by preparing a reaction mixture
composed of 30 mM CH_3COOK, 360 µM $MgCl_2$, 30 µM amino acid mix
minus methionine, 1µg of RNA, reticulocyte lysate 10 µl, 90 mM

DTT, radioactively labelled methionine 2 µl (Amersham, Cat.
No.:SJQ0079), RNasin (Promega, Cat. No.: N2511) 1 µl and water
to 33 µl. The TnT system was used with linear DNA in a
reaction mixture composed of 10 µl of TnT reticulocyte lysate,
5   30 mM CH3COOK, 360 µM MgCl2, 30 µM aminoacid mix minus
methionine, 1µg of DNA, 21 mM DTT, radioactively labelled
methionine 2 µl, RNasin 1 µl and water to 33 µl.

Radiolabelled proteins were separated on SDS-page. (Sambrook,
10   J., Fritsch E,.F., Maniatis, T., (1989). Molecular cloning, a
laboratory manual. Cold Spring Harbor Laboratory Press). Gels
were fixed in destaining solution for 30 minutes, followed by
30 minutes soaking in Amplify (Amersham, Cat. No.: NAMP 100)
under gentle shaking, dried on 3 MM paper and subjected to
15   autoradiography.

Using the IVT methodology, N-terminally truncated versions of
NS2/3 from the HCV J-strain starting with amino acids 903,907,
913 and 919 all ending at amino acid 1206 (Construct numbers
20   25, 28, 31 and 33 from Example 1) were compared in IVT to the
construct number 19, having the native N-terminus.

An autoradiogram of labelled products expressed from NS2/3
constructs was obtained. The autoradiogram was prepared as
25   follows; NS2/3 constructs were produced by in vitro
translation in the presence of ^{35}S labelled methionine using
the TnT T7 Quick Coupled Transcription/Translation System.
After 1 hour incubation at 23 C reactions were stopped by the
addition of SDS sample buffer and analysed by SDS 12.5 % PAGE.
30   Gels were soaked with AmplifyTM and analysed by
autoradiography.

The experiment showed that N-terminal deletions up to residue

907 are tolerated without impairment of the catalytic activity
of the NS2/3 protease. The truncated constructs offer the
advantage that they are devoid of a large hydrophobic
N-terminal portion that could cause aggregation of the protein
5 during heterologous expression.

EXAMPLE 3
Transient expression of NS2/3 protease construct in eukaryotic
cells.

10

The activity of the HCV NS2/3 protease and its inhibition was
investigated using transient expression in eukaryotic cells.

Hep3B or HeLa cells are well suited for this purpose. HeLa
15 cells are seeded at a density of 6 x 10^5 cells/plate and
infected with vaccinia virus vTF7-3 at a multiplicity of 5 PFU
per cell. This infection will lead to the expression of T7 RNA
polymerase in the cells and can be used to express proteins
under the control of a T7 RNA polymerase promoter. The method
20 is extensively described in Tomei et al, (1993) J. Virol. 67,
4017 and Kohara et al, (1992) J. Gen. Vir. 73: 2313-2318.

After adsorption for 30 minutes at 37°C, 3 ml of Dulbecco's
modified Eagle's MEM supplemented with 10% fetal calf serum
25 were added. Cells were incubated for an additional 30 minutes
at 37°C. 20 µg of recombinant plasmid containing NS2/3
protease constructs according to construct numbers 16-35 are
precipitated in calcium phosphate as described in Sambrook et
al (1989), and added directly to each plate in a 500 µl
30 volume.

At 4 hours post-transfection the medium was replaced with MEM
lacking methionine (Gibco Cat No.: 31900-020) and the cells

were starved for 1 hour at 37°C. Cells were then radiolabelled
for 3 hours with 400 µCi of Tran^{35}S label (ICN Cat No.:51006)
in 2 ml MEM lacking methionine and supplemented with 2%
dialyzed fetal calf serum. Cells were harvested and

5   resuspended in 20 mM Tris pH 8.0, 150 mM NaCl, 1% Triton
X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 1 mM
dithiothreitol.

NS2/3 protease expression and activity can be determined in

10 cell extracts upon immunoprecipitation with specific antisera.
To this purpose, sodium dodecyl sulphate and dithiothreitol
are added to the cell extract to final concentrations of 2%
and 10 mM, respectively. The lysates were then incubated at
room temperature for 1 hour and heated to 95°C for 10 minutes.

15 10 µl of antisera were pre-adsorbed for 1 hour at 4°C in a 400
µl volume of 20 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100
with vT7F3-infected HeLa cell extracts spotted on
nitrocellulose filters.

20 The antibody suspension was then incubated with 60 µl of
protein A Sepharose for 1 hour at 4°C. The resin was pelleted
by centrifugation, washed three times in 20 mM Tris pH 8.0,
150 mM NaCl, 1% Triton X-100 and resuspended in 400 µl of the
same buffer. 20 µl of cell lysate were added to the resin and

25 incubated for 1 hour at 4°C.

The protein A sepharose suspension was subsequently layered on
0.9 ml of 5 mM Tris pH 7.4, 16.5 mM EDTA, 0.1 % sodium
deoxycholate, 0.25 % Nonidet P-40, 30% (w/v) sucrose and

30 pelleted by centrifugation in a microcentrifuge at room
temperature. The pellet was washed twice with the same buffer
in the absence of sucrose and once with water. Samples were
analysed by autoradiography upon separation by SDS-

polyacrylamide electrophoresis.

EXAMPLE 4
Expression in *E. coli* in the presence and absence of Zn ions.

Several systems for the heterologous expression of active
NS2/3 protease are known in the art. In the present example
E.coli was used for the expression of NS2/3 protease. These
cells were also driven to express the NS2/3 protein in an
uncleaved form according to procedure outlined below. Other
bacterial or eukaryotic expression systems may be used for the
same purpose.

pT7-7 vectors containing truncated NS2/3 protease sequences
according to construct numbers 1-15 were used to transform *E.
coli* BL21 (DE3). Cells were grown in M9 minimal medium
supplemented with 50 µM biotin, 2.4 µg/ml thiamine, 3.4 µg/ml
FeSO4 and 200 µM Zn Cl_2 at 37°C to an optical density at 500 nm
of 0.8. Temperature was decreased to 23°C and protein
induction was initiated by the addition of 200µl IPTG. After 3
hours, cells were harvested by centrifugation.

Cell extracts may be analysed by polyacrylamide gel
electrophoresis run under denaturing conditions. This
experiment provided evidence for the efficient induction of
NS2/3 precursor protein in addition to two proteins migrating
with the apparent molecular weights of the expected NS3·and
NS2 cleavage products.

Since the NS2/3 precursor undergoes substantial self-
processing during its induction, it is clearly not possible to
obtain large amounts of pure, uncleaved precursor protein
under these conditions. Two strategies were therefore

attempted to decrease or halt the self processing reaction
during protein induction.

1.   Induction at low temperature.

5 Induction at 15°C decreased the total amount of protein
produced but only slightly affected the relative extent of
self-processing. A further decrease of temperature leads to a
severe impairment of protein production. It therefore
appeared that the relative amount of potentially purifiable

10 precursor cannot be increased through the modulation of
temperature during induction.

2.   Induction in minimal medium with low zinc content.
The NS3 protein is known to contain stoichiometric amounts of

15 zionc which are required for its folding and stability (De
Francesco et al, 1996). If the NS3 protein is induced in *E.
coli* grown in minimal medium in the absence of added zinc, it
will not fold properly and forms aggregates known as inclusion
bodies. The truncated NS2/3 protease constructs will drive

20 the formation of insoluble protein if expressed in E. coli
grown in M9 minimal medium not supplemented with zinc ions.
Under these conditions, most of the NS2/3 protein is found as
an uncleaved precursor and only very low amounts of NS3
cleavage product are visible on a Coomassie-stained gel. This

25 procedure therefore provides a way of expressing the NS2/3
protease in a latent, uncleaved form.
In summary, to obtain a catalytically competent NS2/3
precursor in a latent, inactive form which is potentially
amenable for purification, induction of the protein was

30 performed in minimal growth medium in the absence of zinc.

EXAMPLE 5

58

<u>Purification of the unprocessed NS2/3 protease from inclusion</u>
<u>bodies</u>.

To obtain NS2/3 protease in a pure form from bacterial cells,
5   *E. coli* cells were grown in minimal medium in the absence of
added zinc ions and were induced to produce NS2/3 proteins
using construct numbers 1-15 (see Example 1), as outlined
above.

10 The cells were harvested by centrifugation and the cell pellet
was washed with PBS buffer (25 mM sodium phosphate pH 7.5, 140
mM NaCl). The washed pellet was next resuspended in lysis
buffer (40 ml/l of growth medium) containing 25 mM sodium
phosphate pH 6.5, 3 mM DTT, 500 mM NaCl, 0.5 % CHAPS and 15%
15 glycerol. Upon disruption of bacterial cell walls using a
French pressure cell, 10 mM $MgCl_2$ was added to the homogenate
which was then incubated for 30 minutes at 4°C in the presence
of 6 U/μl DNase. The homogenate was centrifuged for 15 minutes
at 12000 xg.

20

The pellet of the centrifugation contained the NS2/3 protein
in addition to other bacterial protein contaminants. It was
washed twice with lysis buffer, once with lysis buffer
supplemented with 1% NP-40 and once with 20 mM sodium
25 phosphate pH 7.5, 3 mM DTT. The final pellet typically
contained an 80% pure NS2/3 protein.

The protein may be further purified using the following
procedure. The pellet was resuspended in 7 M guanidine
30 hydrochloride, 25 mM Tris pH8.7, 100 mM DTT and loaded on a
26/60 Superdex 75 gel filtration column (Pharmacia)
equilibrated with 6 M guanidine hydrochloride, 25 mM Tris pH
7.5, 3 mM DTT, 150 mM NaCl and operating at a flow rate of 2

ml/min. The fractions containing NS2/3 are pooled and loaded
on a 0.5 x 20 cm Source 15RPC reversed phase chromatography
column (Pharmacia) equilibrated in 90 % H20, 0.1% TFA (solvent
A) and 10% acetonitrile 0.08% TFA (solvent B).

A gradient from 10% B to 90% B in 1 hour at a flow rate of 4
ml/min was used to elute the NS2/3 protein in a pure form from
the column. The NS2/3 containing fractions were pooled,
lyophilized and the protein was resolubilized in 7 M guanidine
hydrochloride, 25 mM Tris pH 8.7, 100 mM DTT.

Typically, the protein had a purity >95% and was obtained with
a yield >2mg/l of bacterial culture. The purified protein was
characterized by electrospray mass spectrometry, thereby
verifying that no modifications of the protein took place
during the expression or purification procedure.

The mass found by this technique for NS2/3 H6-907-1206-ASK4
was 33694 Da and corresponded to its theoretical mass
calculated from the amino acid composition. This documents
that no detectable modification of the proteins had occurred
during this purification procedure. This notion is further
corroborated by N-terminal amino acid sequence analysis done
using Edman degradation on a gas-phase sequencer. This
analysis gave the expected N-terminal sequence M-H-H-H.

The yields of pure protein that were obtained varied
considerably among the different constructs 1-15 listed in
Example 1. The highest levels of bacterial protein expression
were obtained with the His + Lys tagged constructs using *E.
coli* BL-21 cells. The expression of the NS2/3 907-1206 ASK$_4$-
based mutant constructs was very low and had to be optimised.
For these constructs, the highest levels of expression were

obtained using *E. coli* B834 cells and minimal medium.

EXAMPLE 6

Refolding of the purified NS2/3 protease

5

A systematic screen for re-folding conditions was set up,
varying the following parameters: re-folding methodology
(dialysis, rapid dilution, stepwise dilution), protein
concentration (10 - 100 µg/ml), pH (6-9), ionic strength (25-
10 275 mM), polar additives (. 0.5 M arginine), non-polar
additives (. 20% glycerol, 1% sucrose), residual chaotrope
concentration (0- 0.75 M guanidine), detergent (. 2% CHAPS),
PEG 4000 (. 0.05%), temperature (4 - 23°C) and Zn^{++}
concentration (30-100 µM). In all of the experiments, the
15 recovery of soluble precursor upon ultracentrifugation was
monitored.

Using this methodology, the following factors were identified
as being important for the recovery of soluble protein after
20 refolding: removal of chaotrope by dialysis, protein
concentration < 100 µg/ml, residual chaotrope concentration of
0.75 M guanidine in the refolding buffer, ionic strength > 200
mM NaCl. Under suitable refolding conditions, more than 80% of
the refolded precursor may be found in the supernatant after
25 120K xg ultracentrifugation.

From this two methods were developed that can be used for
different purposes. Method A allows to generate a refolded
protein at micromolar concentrations that does not undergo
30 cleavage unless transferred into an appropriate buffer,
capable of sustaining the cleavage reaction. Method B allows
the refolding of unfolded NS2/3 protein in a buffer that
simultaneously sustains the cleavage reaction.

Method A. 200 μl of a solution containing 3 μM NS2/3 protease
polypeptide in 6 M guanidine hydrochloride, 25 mM Tris pH 8.7,
100 mM DTT are dialysed against 20 ml of 50 mM Tris pH 7.5, 50
5 μM Zn Cl2, 3 mM DTT, 250 mM NaCl, 750 mM guanidine
hydrochloride using a SpectraPor dialysis membrane with a
cut-off of 10 kDa. The dialysis is performed at 4°C. After two
hours the protein solution can be withdrawn, aliquoted and
shock-frozen in liquid nitrogen. No cleavage occurs during
10 this dialysis and the protein can be activated to undergo
cleavage upon addition to a buffer that activates the cleavage
reaction as outlined below.

Method B. A solution of 5 μM NS2/3 protease polypeptide in 6 M
15 guanidine hydrochloride, 25 mM Tris pH 8.7, 100 mM DTT is
diluted 50-fold into a buffer containing 50 mM Tris pH 7.5, 3
mM DTT, 50% glycerol, 1% CHAPS, 50 μM ZnCl2, 250 mM NaCl at a
temperature of 4°C. After 5 minutes the temperature is raised
to 23°C thereby initiating the cleavage reaction.
20

For both methods A and B, variations of the composition of the
buffers and of the protein concentration are possible and
their consequences are outlined in more detail below. The
yield of refolded, active protein varies between 30% and 80%
25 and depends on the buffer composition, the amino acid
composition of the NS2/3 protein and its purity.

EXAMPLE 7
Detecting the *in vitro* activity of the purified NS2/3 protease
30

Proteins refolded according to method A were diluted at least
5-fold into a buffer having the following composition
(activity buffer): 50 mM Tris pH 7.5, 3 mM DTT, 50% glycerol,

1% CHAPS, 50 µM ZnCl2, 250 mM NaCl.

At timed intervals, aliquots of the activity buffer containing
refolded NS2/3 protease were withdrawn, the reaction was
5   stopped by addition of 0.1% sodium dodecyl sulfate and samples
were loaded on a 12.5 % sodium dodecyl polyacrylamide gel and
analysed upon electrophoresis by silver stain or by Western
blot.

10 Proteins refolded according to Method B were simply incubated
at 23°C for up to two hours and analysed in the same way.

After two hours of incubation under these conditions, an
intense band, migrating with the molecular weight of truncated
15 NS3 was detected on Western blots stained with anti NS3
antibodies.

On silver stained SDS-PAGE two bands corresponding to the Mw
of the truncated NS2 and NS3 proteins were detected in
20 addition to the uncleaved precursor. About 30% of the
precursor protein was estimated to have been processed in this
experiment.

A more convenient and quantitative format of an NS2/3 protease
25 assay involves the separation of precursor and products by
HPLC. The following method was developed: Proteins refolded
according to method A or B were incubated in activity buffer
at 23°C. At timed intervals 200 µl-aliquots were withdrawn and
20 µl 10% TFA were added to stop the reaction. The solution
30 was injected on a Poros R1/H perfusion chromatography column
(4.6 mm x 50 mm, PerSeptive Biosystems, Cat No.: 1-1014-24)
equilibrated with 90% H20/0.1%TFA (buffer A) and 10%
acetonitrile/0.08% TFA (buffer B).

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The column was operated at a flow rate of 2.5 ml/min using a
Merck-Hitachi high performance liquid chromatograph equipped
with a fluorescence detector. A gradient from 10% - 90% B in
15 minutes was used to separate the precursor from its
5 cleavage fragments. Using the monitoring of tryptophan
fluorescence (excitation 280 nm, emission 350 nm) less than 5
nM protein can be reliably detected and quantified by peak
integration.

10 Figure 2 shows two typical chromatograms recorded at time zero
and upon overnight incubation of NS2/3 H6-907-1206-ASK4
(Construct Number 3) in activity buffer. The peak marked "NS3"
co-migrated with a protein standard encompassing amino acids
1027-1206 and carrying the C-terminal extension ASKKKK. To
15 further verify that during the auto-cleavage reaction of
purified NS2/3 protease processing occurred at the authentic
cleavage site, the NS3 cleavage product.was isolated and
characterized using N-terminal sequence analysis by Edman
degradation. The sequence obtained was A-P-I-T, that
20 corresponds to residues 1027-1030 of the HCV polyprotein and
unambiguously indicated that cleavage had occurred at the
authentic NS2/3 site.

We estimated a first order rate constant of 0.06 min^{-1} for the
25 cleavage reaction and a t/2 of 11.5 minutes. These data are
not dissimilar from published data on the cleavage reaction of
full-length NS2/3 constructs in an *in vitro*
transcription/translation system, in which t/2 of 10-15
minutes and a maximum cleavage of 70% were found.
30
Using several different enzyme preparations, we obtained
between 15 and 80% of cleavage and rates of 0.03 min^{-1} - 0.07
min^{-1}. The cleavage fragments were submitted for N-terminal

sequence analysis to verify that cleavage occurred at the
authentic cleavage site.

The NS3 protease activity of the refolded NS2/3 H_6-907-1206-
5 ASK_4 precursor was also investigated. No significant change in
NS3 protease activity was noticed during an auto-cleavage
reaction which led to 30% of processing after two hours of
incubation. The extent of NS3 protease activity was compatible
with the presence of about 25% of active molecules, assuming
10 that the specific activity of the NS3 protease is unchanged in
the NS2/3 H_6-907-1206-ASK_4 precursor.

These experiments show that the amount of catalytically
competent protein that can be recovered has both NS2 and NS3
15 protease activities. The remainder of the protein is probably
mis-folded.

EXAMPLE 8
Characterization of the activity of the NS2/3 protease
20
The HPLC method for detection of cleavage products arising
upon incubation of the purified and refolded NS2/3 protease in
activity buffer can be used to quantify the kinetic parameters
of the reaction and to determine the influence of different
25 physico-chemical conditions on its rate.

Figure 3 shows a typical cleavage time course. 30 nM NS2/3
protease were incubated in 50 mM Tris pH 7.5, 3 mM DTT, 50%
glycerol, 1% CHAPS, 50 μM $ZnCl_2$, 250 mM NaCl and the cleavage
30 reaction was followed by measuring the amount of NS3 cleavage
product formed with time. This was done by integrating the
NS3 HPLC peak area.

In Figure 3, left panel, a plot of NS3 peak area versus time
is shown. The data points can be best fitted with a single
exponential equation, allowing the assignment of an apparent
rate constant. It was found that this rate constant is
5   affected by the variation of several parameters such as ionic
strength, glycerol concentration, pH, detergent concentration.

An unexpected finding is the fact that the rate constant also
shows a protein-concentration dependency (Figure 3, right
10   panel). This is indicative of a multimer being the active
species. In fact, gel filtration chromatography showed
evidence for a dimeric species being formed in solution.
Dimerization is a novel property of the NS2/3 protease that
could be used to develop a strategy aimed at finding
15   inhibitors of NS2/3 that interfere with dimer formation.

EXAMPLE 9

Assays for inhibitors of the NS2/3 protease

20   The invention also provides methodologies that can be used to
identify inhibitors of the cleavage reaction. Such inhibitors
can be found by screening compound collections or
combinatorial libraries for NS2/3 protease inhibitory
activity. Conveniently, small organic molecules are
25   solubilized in DMSO and added to the assay. In all of the
following assays the addition of up to 10% DMSO is tolerated
without substantial impairment of NS2/3 protease cleavage
activity.

30   The following methods are examples of assay methods that may
be used:

A. Cell-based assay

Expression plasmids suitable for transient expression of NS2/3
protease in eukaryotic cells (pCITE construct numbers 16-35;
Example 1) are described herein. These plasmids contain the
NS2/3 protease under the control of a T7 RNA polymerase
promotor. The use of this system to monitor expression of
heterologous proteins in eukaryotic cells and -more
specifically- to monitor the activity of viral proteases is
well known in the art, (Tomei, L. *et al.* (1993). J. Virol. 67,
4017.) and described in Example 3.

Inhibition of NS2/3 protease activity by externally added
molecules results in decreased levels of cleavage products
that can be determined upon radiolabelling of proteins and
isolation of NS2/3 protease and its NS3 cleavage product by
immunoprecipitation with anti NS3 antisera, using established
methods known in the art.

B. In vitro translation assay

Active NS2/3 protease can be generated using a coupled in
vitro transcription/translation system or by in vitro
translation of the appropriate RNA molecules as outlined above
(Example 2).

The invention provides plasmids suitable for T7 RNA polymerase
driven production of NS2/3 protease encoding RNA molecules
according to procedures known to those skilled in the art.
Inhibitors are added to the reaction mixture and their potency
determined by titration experiments.

Radiolabeling of proteins by addition of radioactively
labelled amino acids and the subsequent analysis by SDS-PAGE
and autoradiography allows monitoring of the inhibition of

NS2/3 protease activity by a decrease in the amount of
radioactive cleavage products.

C. Cleavage detection by SDS Page or HPLC

5 NS2/3 protease constructs provided by this invention are
purified and refolded according to the methods of this
invention and incubated in 200 µl of activity buffer (50 mM
Tris pH 7.5, 3 mM DTT, 50% glycerol, 1% CHAPS, 50 µM $ZnCl_2$,
250 mM NaCl) or another buffer suitable for sustaining the
10 cleavage activity. Typically, 30 nM to 300 nM NS2/3 protease
are used in the assay and incubated in the appropriate buffer
in the absence or in the presence of up to 10% (v/v) of a
solution of organic compounds in DMSO. At the lower the
protein concentration the NS2/3 protease is present in the
15 solution both as active dimer and as inactive monomer. This
leads to a slower overall reaction rate.

The use of protein concentrations similar to or below the
equilibrium dissociation constant of the dimer may be
20 advantageous if one wishes to find inhibitors of dimer
formation. Furthermore, low protein concentration may also be
required to determine the activity of very potent inhibitors.
At protein concentrations above 100nM most of the protein will
be present as active dimer and the cleavage reaction will be
25 faster than at low protein concentration (see also Figure 3).

Depending on the protein concentration used in the assay, the
reaction is allowed to proceed for 10 to 30 minutes and
stopped either by inactivating the NS2/3 protease through the
30 addition of 20 µl 10% TFA (if the cleavage is detected by
HPLC) or of SDS sample buffer (if cleavage is detected by SDS
PAGE). In each case, the reaction is stopped in the linear
range of the time course curve (Figure 3). This is important

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since deviation from linearity may lead to an underestimation of the potency of added inhibitors.

Subsequently, the amount of cleavage product formed in the absence of inhibitor is compared to the amount of the same cleavage product formed in the presence of added inhibitor. The % inhibition is calculated from this comparison and determined at different inhibitor concentrations, leading to an accurate determination of the potency of the inhibitory compound. Either the generation of the NS3 or the NS2 cleavage fragment can be monitored to this end.

Using SDS PAGE as detection method, it is convenient to perform a Western blot stained with either NS2 or NS3-specific antisera to improve sensitivity. The intensity of the bands can be estimated by densitometry or other imaging techniques known in the art.

The preferable detection method is HPLC that allows the quantitative detection of uncleaved NS2/3 and both the NS2 and NS3 cleavage fragments within a few minutes. The assay can be used to screen collections of chemical compounds using robotic sample assembly and analysis. An example of an HPLC run documenting the separation and quantification of NS2/3, NS2 and NS3 is shown in Figure 2.

D. High throughput screening assay
Large compound collections or combinatorial libraries often containing >10^5 distinct chemical entities are promising sources for lead compounds in a drug development program. Handling of such large numbers of compounds requires robotic technologies and an assay that can be performed in a microplate format. The invention therefore also provides

methods to assay NS2/3 protease activity in a microplate
assay.

The assay, which is outlined in Figure 1, was performed as

5 follows: A six-histidine tag was engineered at the N-terminus
of NS2/3 protease polypeptide. The tagged protein was purified
as specified above and refolded following the refolding
procedures A or B. For the sake of simplicity, refolding
procedure B was preferred, whereby denatured, histidine tagged

10 NS2/3 protease polypeptide was diluted into 100 µl activity
buffer (50 mM Tris pH 7.5, 3 mM 2-mercaptoethanol, 50%
glycerol, 1% CHAPS, 50 µM $ZnCl_2$, 250 mM NaCl) at a final
concentration of 50 nM.

15 Incubation for 15 minutes at 23°C results in partial
processing of the NS2/3 protease. Incubation time was chosen
in order to obtain about 10% of processing. Only the NS2
cleavage fragment and the uncleaved NS2/3 precursor possess
histidine tags. These histidines may be captured by the

20 addition of a metal affinity resin. Hence, after the
incubation period 100 µl of a 50 % (v/v) slurry of a Talon
metal affinity resin (Clontech, Cat No.: 8901-3) equilibrated
in 50 mM Tris pH 7.5, 250 mM NaCl were added. The resin
sequestered the histidine-tagged species and left the untagged

25 NS3 molecule in solution.

The NS3 molecule generated during the processing reaction of
the NS2/3 protease has a serine protease activity. This
activity may be used as readout of the processing reaction

30 between NS2 and NS3. In fact, the catalytic activity of NS3
will amplify each NS2/3 processing event, e.g. through
turnover of a fluorogenic NS3 substrate.

After sedimentation of the resin a 40 µl aliquot of the
supernatant was withdrawn and added to a well of a second
96-well microplate containing 200 µl 50 mM Tris pH 7.5, 0.1%
Triton X-100, 10 mM DTT, 15% glycerol, 150 mM NaCl and 20 µM
Pep4AK, having the sequence KKKGSVVIVGRIILSGR-NH2. Pep4AK is
a co-factor of the NS3 protease and was added in order to
obtain maximum activity.

At this point, 5 µM of an internally quenched fluorogenic NS3
substrate having the sequence Mca-DDIVPCSMSK[DNP] was added.
The reaction was followed using a fluorescence microplate
reader (excitation 325 nm, emission 393 nm).

Robotic handling of all the steps required for this assay is
possible, allowing screening of a large number of compounds in
a short time period. Furthermore false positives that could
arise due to inhibition of the NS3 protease are minimized due
to the tenfold dilution of the original solution during the
NS3 protease assay.

CLAIMS

1.   A polypeptide consisting of a fragment of an HCV NS2/3
protease, which protease is produced naturally as an HCV

5   NS2/3 protease precursor, wherein the fragment has as its N-
terminal residue an amino acid that is at a position from
residue 903 to 913 in the HCV NS2/3 protease precursor and as
its C-terminal residue an amino acid that is at a position
from residue 1206 to 1657 in the HCV NS2/3 protease

10 precursor, which polypeptide when in a homodimer has auto-
proteolytic activity.

2.   A polypeptide according to claim 1 wherein the C-
terminal residue of the fragment is the amino acid that is at

15 position 1206 in the HCV NS2/3 protease precursor.

3.   A polypeptide according to claim 2 wherein the N-
terminal residue of the fragment is the amino acid that is at
position 903 in the HCV NS2/3 protease precursor.

20
4.   A polypeptide according to claim 2 wherein the N-
terminal residue of the fragment is the amino acid that is at
position 907 in the HCV NS2/3 protease precursor.

25 5.   A polypeptide consisting of an amino acid sequence that
has 90% sequence identity over its length compared with a
fragment of an HCV NS2/3 protease, which protease is produced
naturally as an HCV NS2/3 protease precursor, wherein the
fragment has as its N-terminal residue an amino acid that is

30 at a position from residue 903 to 913 in the HCV NS2/3
protease precursor and as its C-terminal residue an amino
acid that is at a position from residue 1206 to 1657 in the
HCV NS2/3 protease precursor, which polypeptide when in a

homodimer has auto-proteolytic activity, and wherein the HCV
NS2/3 protease precursor is as encoded by nucleic acid within
HCV J strain (Genbank Acc. No. D90208), HCV H strain (Genbank
Acc. No. M67463) or HCV H77 strain (Genbank Acc. AF009606).

6.   A polypeptide according to any one of claims 1 to 5
fused to one or more heterologous amino acid residues.

7.   Isolated nucleic acid encoding a polypeptide according
to any one of claims 1 to 6.

8.   An expression vector comprising nucleic acid according
to claim 7 operably linked to regulatory sequences for
expression of said polypeptide.

9.   A host cell transformed with an expression vector
according to claim 8.

10. A method of producing a polypeptide, the method
comprising causing expression from an expression vector
according to claim 8 to produce said polypeptide.

11. A method according to claim 10 comprising culturing host
cells transformed with said expression vector under
conditions for production of said polypeptide.

12. A method according to claim 11 wherein the host cells
are cultured in conditions whereby the polypeptide produced
accumulates in insoluble inclusion bodies.

13. A method according to claim 12 wherein the host cells
are cultured in zinc-depleted medium.

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14.   A method according to any one of claims 10 to 13
comprising isolating and/or purifying said polypeptide.

15.   A method according to any one of claims 10 to 14
5   comprising renaturing or refolding the polypeptide.

16.   A method according to claim 15 comprising forming the
polypeptide into a homodimer that has auto-proteolytic
activity.

10
17.   A homodimer obtained by a method according to claim 16.

18.   A homodimer of two polypeptides according to any one of
claims 1 to 6.

15
19.   A method of obtaining a polypeptide consisting of a
fragment of an HCV NS2/3 protease, which protease is produced
naturally as an HCV NS2/3 precursor, and which polypeptide
when in a homodimer has auto-proteolytic activity, the method
20   comprising:
        providing one or more truncated fragments of the HCV
        NS2/3 protease;
        testing the truncated fragment or truncated fragments
        for ability to form a homodimer that has auto-
25   proteolytic activity, to identify a homodimer that has
        auto-proteolytic activity;
        whereby said polypeptide is obtained.
20.   A method according to claim 19 wherein the truncated
fragment has or truncated fragments have as N-terminal
30   residue an amino acid that is at a position from residue 903
to 913 in the HCV NS2/3 protease precursor and as C-terminal
residue an amino acid that is at a position from residue 1206
to 1657 in the HCV NS2/3 protease precursor.

21.   A method according to claim 19 or claim 20 comprising
production of said truncated fragment or truncated fragments
by expression from encoding nucleic acid.

5

22.   A method according to claim 21 wherein said truncated
fragment or truncated fragments are produced by expression
caused by culturing host cells transformed with said encoding
nucleic acid under conditions for production of said

10   truncated fragment or truncated fragments.

23.   A method according to claim 22 wherein the host cells
are cultured in conditions whereby the polypeptide produced
accumulates in insoluble inclusion bodies.

15

24.   A method according to claim 23 wherein the host cells
are cultured in zinc-depleted medium.

25.   A homodimer of two polypeptides obtained by a method

20   according to any one of claims 19 to 24.

26.   An assay method for testing ability of an agent to
modulate activity of HCV NS2/3 protease, comprising:
(a)   bringing a test agent into contact with polypeptides

25   according to any one of claims 1 to 6 or a homodimer of
             two said polypeptides; and
(b)   determining formation of homodimer of two said
             polypeptides and/or HCV NS2/3 protease activity.

30   27.   A method according to claim 26 comprising determining
dimerisation of said polypeptides.

28.   A method according to claim 26 or claim 27 comprising
determining HCV NS2/3 protease activity.

29.   A method according to any one of claims 26 to 28
5   comprising identifying a test agent as a substance which
modulates activity of HCV NS2/3 protease.

30.   A method according to claim 29 further comprising
formulation of said substance which modulates activity of HCV
10   NS2/3 protease, or optionally, if said substance is peptidyl,
nucleic acid encoding said substance, into a composition
comprising at least one additional component.

31.   Use of a polypeptide according to any one of claims 1 to
15   6 or a homodimer of two said polypeptides in identifying or
obtaining a substance which modulates HCV NS2/3 protease
activity.

Step 1.   - Refold NS2/3

Step 2.   - Activate cleavage in appropriate buffer
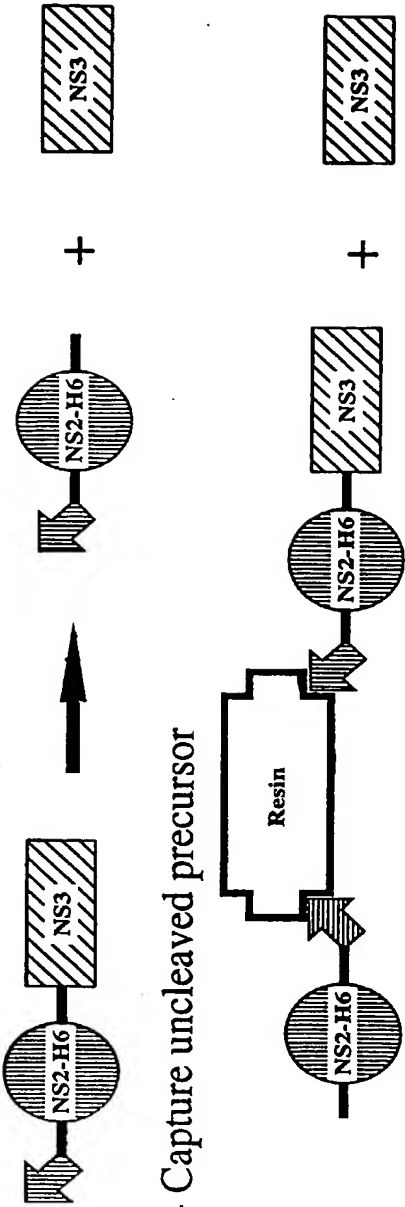
Step 3.   - Capture uncleaved precursor
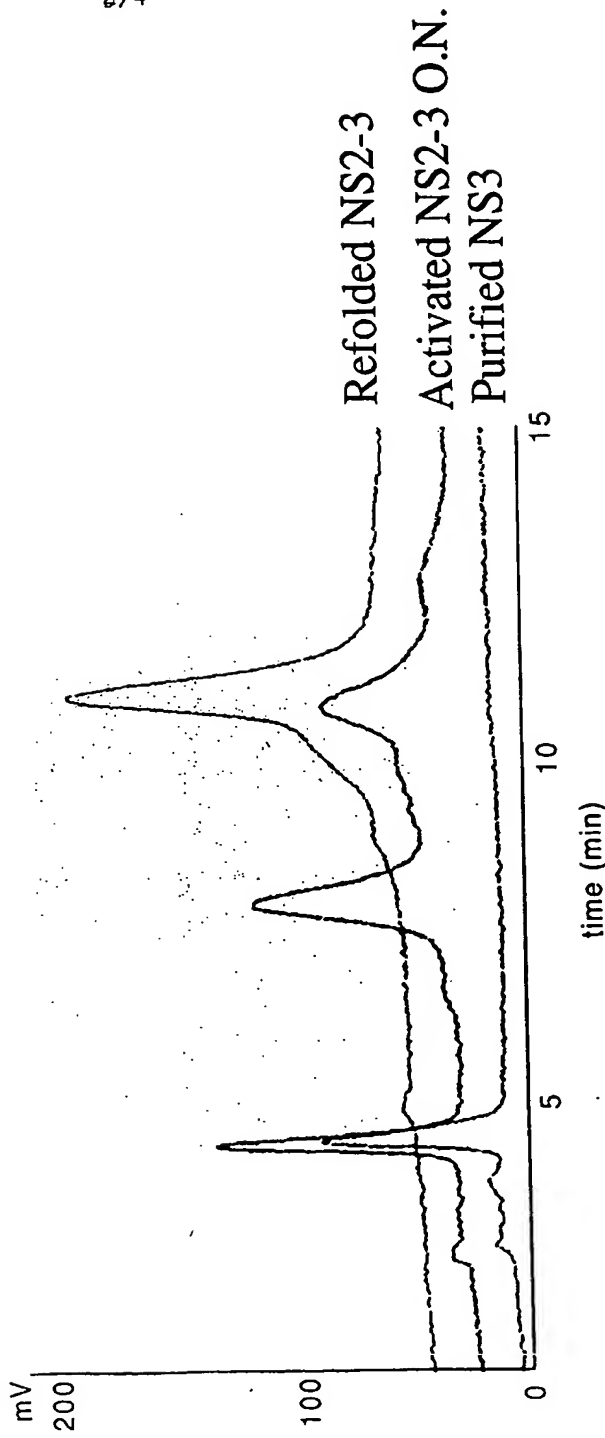
Step 4.   - Measure free NS3 in solution

Figure 1

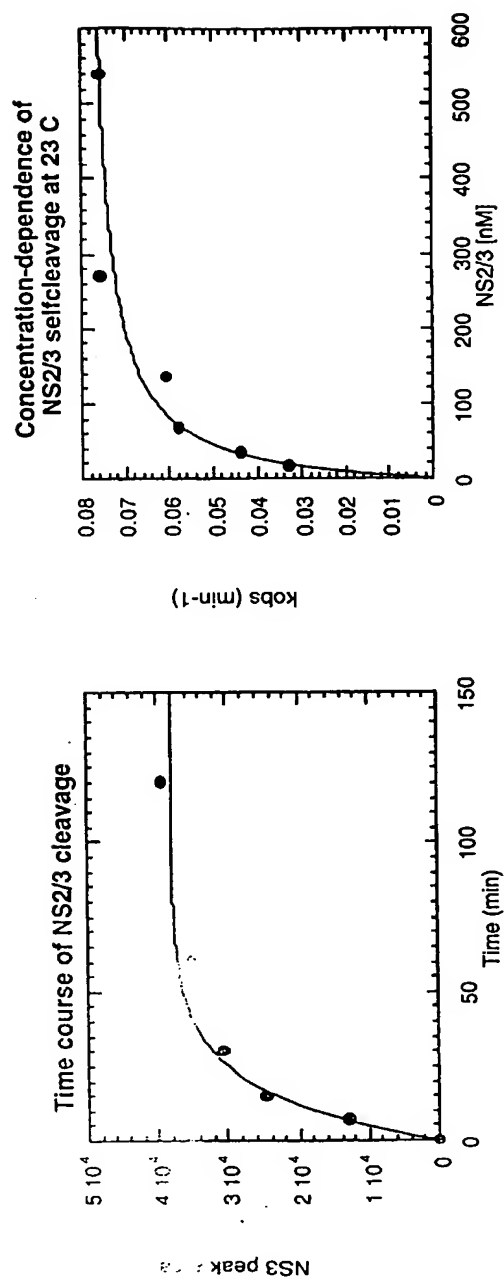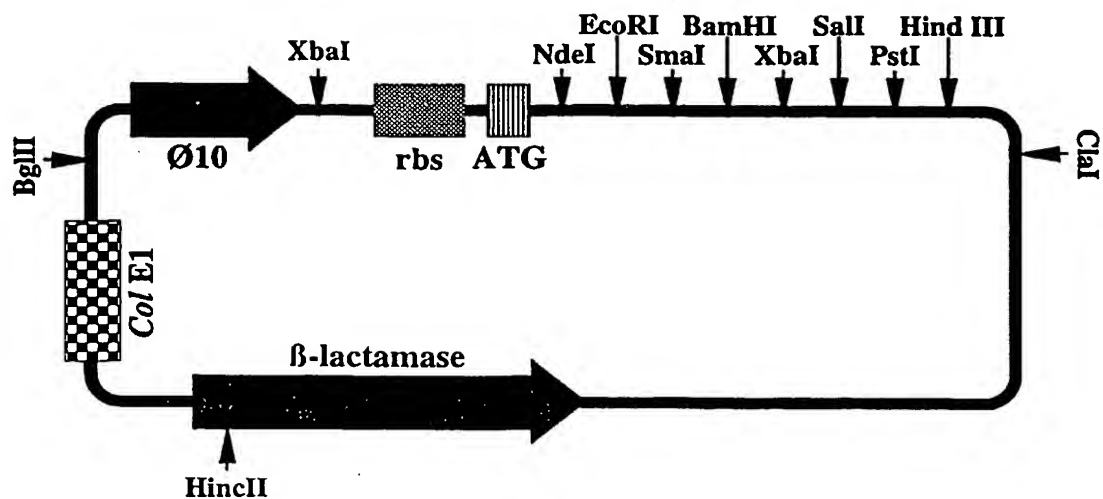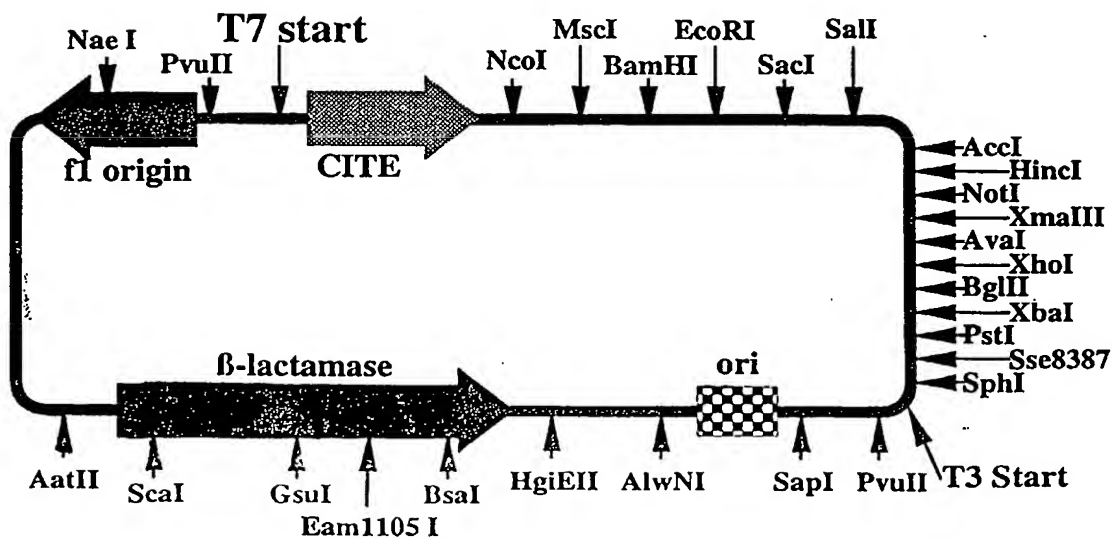Figure 2

Figure 3

4/4

pT7-7

pCITE-2b(+)

Figure 4